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Multiplex and accurate quantification of acute kidney injury biomarker candidates in urine using Protein Standard Absolute Quantification (PSAQ) and targeted proteomics

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ABSTRACT

There is a need for multiplex, specific and quantitative methods to speed-up the development of acute kidney injury biomarkers and allow a more specific diagnosis. Targeted proteomic analysis combined with stable isotope dilution has recently emerged as a powerful option for the parallelized evaluation of candidate biomarkers. This article presents the development of a targeted proteomic assay to quantify 4 acute kidney injury biomarker candidates in urine samples. The proteins included in the assessed panel consisted of myo-inositol oxygenase (MIOX), phosphoenolpyruvate carboxykinase 1 (PCK1), neutrophil gelatinase-associated lipocalin (NGAL) and liver fatty acid-binding protein (L-FABP). The proteomic assay combined an antibody-free sample preparation and a liquid chromatography-selected reaction monitoring (LC-SRM) analysis pipeline. For accurate quantification of the selected candidates, we used PSAQ (Protein Standard Absolute Quantification) standards which are isotopically labeled versions of the target proteins. When added directly to the biological samples, these standards improve detection specificity and quantification accuracy. The multiplexed assay developed for the 4 biomarker candidates showed excellent analytical performance, in line with the recommendations of health authorities. Tests on urine from two small patient cohorts and a group of healthy donors confirmed the relevance of NGAL and L-FABP as biomarkers for AKI diagnosis. The assay is readily adaptable to other biomarker candidates and should be very useful for the simultaneous and accurate quantification of multiple biomarkers.

1. Introduction

Acute kidney injury (AKI) is a common and life-threatening condition with different causes including ischemia, sepsis or nephrotoxic substances. Clinical diagnosis of AKI is currently based on functional biomarkers, mainly serum creatinine, blood urea nitrogen and urine output characterized by a rapid decline in the glomerular filtration rate. Although widely used, these biological parameters provide little information on the underlying cause, the location and extent of kidney damage. In addition, serum creatinine is not sensitive to the loss of kidney reserve. To improve the specificity of diagnosis and detect kidney injury at early stages, intense efforts have been directed

Abbreviations: AKI, acute kidney injury; L-FABP, liver fatty acid-binding protein; MED-FASP, multiple enzyme digestion – filter aided sample preparation; MIOX, myo-inositol oxygenase; NGAL, Neutrophil gelatinase-associated lipocalin; PCK1, phosphoenolpyruvate carboxykinase 1; SRM, Selected Reaction Monitoring; PSAQ, Protein Standard Absolute Quantification

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to the development of novel biomarkers [1]. Several protein biomarker candidates were discovered in animal models of AKI and were subsequently evaluated in established human disease. Among these proteins, neutrophil gelatinase-associated lipocalin (NGAL), liver fatty acid-binding protein (L-FABP), kidney injury molecule 1 (KIM1) and interleukin-18 (IL-18) emerged as the most promising biomarkers for early detection of kidney injury [1,2]. However, none of these biomarkers obtained formal approval from health authorities for clinical use [3,4]. Recently, a clinical assay simultaneously quantifying insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinase 2 (TIMP2) in urine was approved by the Food and Drug Administration for use in patients at risk of developing AKI [5]. However, additional data from independent studies will be necessary before clinical certainty [3]. In the future, nephrologists will probably use combinations of biomarkers to diagnose specific AKI conditions (sepsis, cardiac surgery, toxic insult) [3]. In this context, high performance analytical tools allowing the simultaneous quantification of several biomarker candidates are necessary. Importantly, these tools must be compatible with small urine samples as AKI patients can be oliguric.

During the last decade, targeted proteomics based on liquid chromatography-selected reaction monitoring (LC-SRM) has emerged as a powerful alternative to immunoassays for the parallelized analysis of protein biomarker candidates in biofluids [6,7]. LC-SRM offers specific advantages including exquisite specificity, high sensitivity, high multiplexing capability and reproducibility [8]. In the field of nephrology, few recent studies described the development of LC-SRM assays for the clinical evaluation of putative AKI biomarkers [9–12]. Among these assays, the best multiplexing performance was obtained by Sigdel and coworkers [11]: 35 proteins were simultaneously quantified in urine, enabling the discrimination of the 3 major AKI phenotypes following kidney transplantation.

Targeted proteomics analyses based on LC-SRM are generally performed using a “bottom-up” workflow which involves the digestion of protein biomarker candidates into peptides and the targeted monitoring of signature peptides as candidate surrogates [13,14]. With this method, biomarker candidate concentrations can be determined using stable isotope-labeled standards (peptides, peptide concatemers or proteins) which are spiked into the samples and serve as references [15,16]. To meet the recommendations of health authorities for bioanalytical assay development, the use of PSAQ standards (Protein Standard Absolute Quantification) is advocated [17]. Indeed, because they are full-length isotope-labeled versions of the targeted proteins, PSAQ standards can be added to the biological samples at early stages of the analytical process and they can thus correct for analytical variabilities due to upstream sample handling or incomplete proteolysis (on the condition that they behave similarly to their protein targets during sample processing) [18–21].

The goal of this study was to develop a high performance proteomics pipeline, based on the use of PSAQ standards and LC-SRM, to simultaneously assay several AKI biomarker candidates in small urine samples. The pipeline was tested on extensively studied biomarker candidates, namely NGAL and L-FABP, and two new potential biomarkers selected from literature and expression data: myo-inositol oxygenase (MIOX) and phosphoenolpyruvate carboxykinase 1 (PCK1). MIOX expression is restricted to the proximal tubule epithelial cells [22]. It was recently identified as a potential plasma biomarker in human patients with AKI [23]. In the kidney, PCK1 is specifically expressed in the proximal tubule epithelial cells [22]. Based on this kidney-predominant expression, we hypothesized that PCK1 could leak into the urine following tubular necrosis. Results showed excellent analytical performance of the assay developed, and confirmed the utility of NGAL and L-FABP as biomarkers of AKI.

2. Material and methods

2.1. Urine samples

Urine samples from AKI patients were provided by nephrology departments from Henri Mondor Hospital (Créteil, France) and Tenon hospital (Paris, France). Experiments and research were conducted in accordance with the principles set out in the WMA Declaration of Helsinki. Urine samples were collected as part of clinical studies that were approved by ethical committee and declared at the Commission Nationale de l'Informatique et des Libertés. All patients provided written informed consent. Urine samples were collected, anonymized, rapidly aliquoted and stored at −80 °C. Patients were classified in two categories according to biopsy-proven pathological diagnosis: those with glomerular injury and those with tubular injury (Supplementary Table 1). Some biological samples were analyzed immediately at the clinical chemistry laboratory to determine standard parameters. Urine from healthy donors was also collected and used for analytical developments and to compare with AKI patients.

2.2. Recombinant proteins

Recombinant NGAL, PCK1 and L-FABP proteins were obtained from Abcam (references ab95007, ab119469 and ab82994 respectively). PSAQ standards (isotopically-labeled recombinant proteins) for the four biomarker candidates were synthesized as previously described [24]. Production was scaled-up at Promise Advanced Proteomics (Grenoble, France). PSAQ standards were checked for isotope incorporation (> 99%) and were quantified by amino acid analysis [25] (Supplementary Fig. 1).

2.3. Urine sample preparation

Urine samples were prepared based on an adaptation of the MED-FASP (multiple enzyme digestion – filter aided sample preparation) method [26]. Briefly, after thawing at room temperature, urine (400 µL) was spiked with defined amounts of PSAQ standards, gently mixed and centrifuged at room temperature for 10 min at 4000*g*. The supernatant was collected and concentrated to 100 µL on a 10-kDa cutoff ultrafiltration device (Amicon). Urinary proteins were denatured and reduced on the device in 4 M urea, 50 mM ammonium bicarbonate and 2 mM TCEP. The sample was washed twice with 4 M urea, 50 mM ammonium bicarbonate before performing alkylation in 4 M urea, 50 mM ammonium bicarbonate and 10 mM iodoacetamide. After two additional washing steps, the sample volume was reduced to 25 µL and proteins were digested for 3 h at 37 °C using trypsin/LysC mix (Promega, Charbonnières les Bains, France) at a protein/enzyme ratio of 1:30 (w/w). The urea concentration was reduced to 1 M and digestion was allowed to proceed overnight at 37 °C. Proteolytic peptides were recovered by adding 50 µL of NaCl 0.5 M to the filter and centrifuging for 40 min at 14 000g at room temperature. The peptide digest was purified on a C18 ZipTip device (Thermo Scientific, Courtaboeuf, France) and dried by vacuum centrifugation. Peptides were resolubilized in 10 µL of 2% acetonitrile, 0.1% formic acid, and 6 µL were injected into the LC-system.

2.4. Calibration experiment

Urine samples (400 µL each) were spiked with increasing amounts of surrogate analytes (unlabeled recombinant proteins) and constant amounts of PSAQ standards (20 ng/mL for PCK1, 30 ng/mL for NGAL and 10 ng/mL for FABP1). Zero samples were also constituted. The LLOQ was determined according to the FDA criteria described in the guidelines for bioanalytical method validation (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances). The LLOQ was established as the lowest concentration on

the titration curve that could be measured with a precision (CV) below 20% and an accuracy between 80% and 120%. At the LLOQ, the signal-to-noise ratio was at least 5/1.

2.5. LC-SRM analysis

LC-SRM analyses were performed on a 6500 QTrap hybrid triple quadrupole/ linear ion trap mass spectrometer (AB Sciex, Les Ulis, France) equipped with a TurboV electrospray ion source and operated with Analyst software (version 1.6.1, AB Sciex). The instrument was coupled to an Ultimate 3000 LC-chromatography system (Thermo Scientific). Chromatography was performed using a two-solvent system combining solvent A (2% acetonitrile, 0.1% formic acid) and solvent B (80% acetonitrile, 0.1% formic acid). Peptide digests were first concentrated on a C18 precolumn (Phenomenex, ref: AJ0-8782) before separation on a Kinetex C18 column (2.1 mm x 100 mm, Core-shell 2.6 µm, 100 Å, Phenomenex, ref: 00D-4462-AN). Peptide separation was achieved using a linear gradient from 3% to 35% B in 30 min, and from 35% to 90% B in 10 min at a flow rate of 50 µL/min. MS data were acquired in positive mode with an ion spray voltage of 4300 V; curtain gas was used at 30 p.s.i. and the interface heater temperature was set to 320 °C. Collision cell exit, declustering and entrance potentials were set to 21, 55 and 14 V, respectively. Collision energy (CE) values were calculated using linear equations based on the unlabeled peptide precursor m/z ratios: CE=0.05m/z+5 (Volts) for doubly charged precursors. The same collision energy was used for both labeled and unlabeled versions of each signature peptide. The analyses combined in the same run: (1) a precursor ion scan between 400 and 1000m/z as a survey scan for Information Dependent Acquisition (IDA), (2) an Enhanced Product Ion (EPI) scan with a scan speed of 1000 amu/sec and a dynamic fill time for optimal MS/MS analysis, (3) an SRM acquisition with Q1 and Q3 quadrupoles operating at unit resolution. For scheduled SRM analyses, the acquisition time window was set to 90 s (calibration curve) or 180 s (clinical samples) and the target scan time was set to 2 s or 1.2 s, respectively. Thus, for chromatographic peaks with a mean base width of 20 s, 10 or 17 points were acquired per LC peak. All MS data have been deposited in the PeptideAtlas SRM Experiment Library (PASSEL) (Identifier PASS00885) [27].

2.6. LC-SRM data analysis

LC-SRM data analysis was performed using Skyline software. Peak picking was performed using the mProphet algorithm and the "*second best peak*" model. A Q-value of 0.01 (1% FDR) was set as the cutoff for peptide signal analysis. In addition to peptide signal scoring, all transitions were individually visually inspected and excluded if they were found to be unsuitable for quantification (low signal-to-noise ratio, obvious interference). Unlabeled/labeled peak area ratios were calculated for each SRM transition and were averaged to determine the corresponding peptide ratio. At least two transition pairs were used to determined biomarker concentration. The protein ratio was calculated from the ratios obtained for its signature peptides. Finally, candidate biomarker concentrations were calculated from the average protein ratio and the concentration of PSAQ standard initially added to the sample.

3. Results

3.1. Development of the LC-SRM method

We first selected signature peptides to be used as surrogates for biomarker candidate detection. This selection involved digesting pure recombinant analogues of the four target proteins with LysC/trypsin mix, followed by LC-SRM analysis. Signature peptides were selected based on the following criteria: (i) the sequence had to be specific based on a BLASTP search against the human proteome background in Uniprot database (Supplementary Fig. 2, Supplementary Table 2) and (ii) the corresponding endogenous peptide had to be devoid of post-translational modifications. Following this selection, a LC-SRM method to analyze the four biomarker candidates was optimized in urine matrix. This optimization involved spiking urine samples with the four recombinant analogues and with the four corresponding PSAQ standards before processing. Digested samples were analyzed using LC-SRM to select the most responsive peptides and SRM transitions. For MIOX, only one peptide was adequately detectable by MS in urine matrix. This peptide was common to the two described isoforms. For PCK1, 5 peptides were selected: 2 were specific for isoform 1 (full-length mature protein), while the three others were shared between isoforms 1 and 2. Nevertheless, as isoform 2 is a predicted splicing variant without any experimental validation at the protein level (according to Uniprot database), the 5 selected peptides were considered as signature peptides for full-length mature PCK1. For NGAL, the two selected peptides were common to the two isoforms described. Indeed, the two NGAL isoforms only differ by 6 amino acids at the C-terminal extremity. For L-FABP, the three peptides chosen were strictly specific to this isoform (the FABP protein family includes 10 different isoforms) [28]. The final LC-SRM method could monitor 11 signature peptides (in labeled and unlabeled forms), each with 3 y-ion fragments, leading to an inclusion list of 66 SRM transitions. The liquid chromatography gradient was specifically designed to minimize peptide co-elution, and SRM acquisition was scheduled to enhance detection sensitivity (Fig. 1).

3.2. Sample preparation optimization

For reliable quantification, each analyte and its PSAQ standard must behave similarly during sample preparation and digestion. Two antibody-free biochemical methods were tested for the preparation of urine samples: (i) precipitation with 6% trichloroacetic acid followed by LysC/trypsin digestion or (ii) MED-FASP which corresponds to filter aided sample processing (FASP) with a double enzyme digestion using LysC and trypsin (Fig. 1) [26]. Comparative tests revealed that MED-FASP allowed the most equivalent behavior between biomarker candidates and their labeled standards (Supplementary Fig. 3). By adding urea to the sample and performing reduction/alkylation treatment early in the process, proteins can be completely denatured to equalize the biochemical behavior of the unlabeled proteins (i.e., the recombinant analogue and the endogenous analyte) and their quantification standards. Through this equalization, quantification errors due to slight differences in sequence or structure are expected to be smoothed. This was important for MIOX, NGAL and L-FABP quantification as their respective PSAQ standards contained a N-terminal hexahistidine purification tag. For NGAL quantification, this initial harmonization of structure was also useful in disrupting the three different forms present in urine: monomeric, dimeric (disulfide-bridged) or covalently conjugated to matrix metalloproteinase-9 (MMP9) [4,29]. In these conditions, denatured proteins were also equally accessible to proteases, ensuring more reliable measurements.

3.3. Assessing the performance of the multiplex proteomic assay

To assess the performance of our assay combining MED-FASP and LC-SRM, a multiplexed calibration experiment was set up using urine from a healthy donor as matrix (Fig. 2). For PCK1, NGAL and L-FABP, 6 non-zero calibration points were created by adding a range of amounts of unlabeled recombinant protein and a constant amount of PSAQ standard to urine samples (400 µL). Zero samples, containing only PSAQ standards, were also constituted. All the calibration points were created as full-technical replicates (n=3). The quantities of unlabeled analytes spiked were calculated to cover physiological levels up to the highest pathological concentrations, as defined in previous studies and/or determined by preliminary experiments performed on

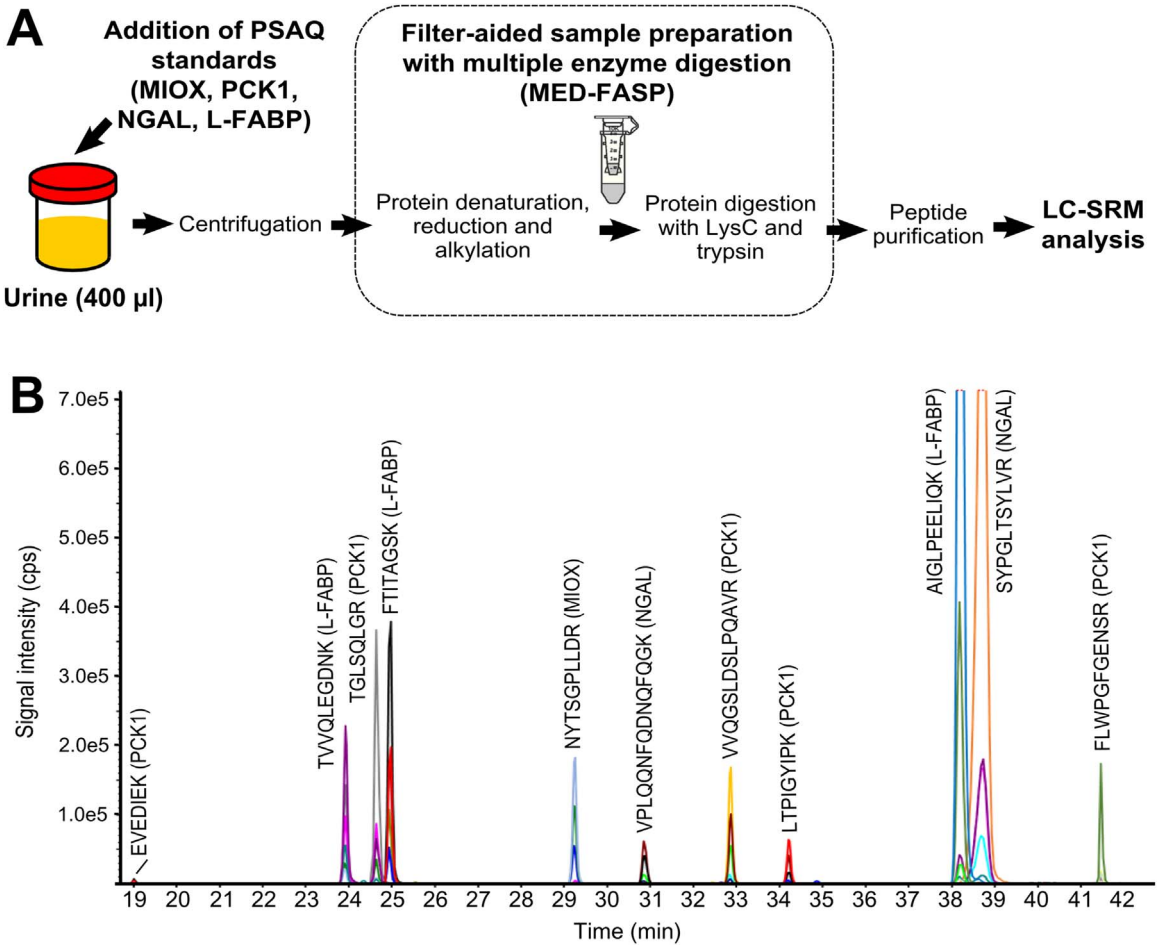

Fig. 1. Analytical pipeline to evaluate AKI biomarker candidates in urine. (**A**) Analytical workflow for the standardization, preparation, digestion and LC-SRM analysis of urine samples. (**B**) Extracted ion chromatogram of a urine sample using scheduled LC-SRM analysis.

urine samples from healthy donors and AKI patients. For MIOX, no exogenous source of surrogate analyte (i.e., an unlabeled recombinant protein) was available, therefore the calibration curve was performed in reverse mode by adding a range of PSAQ standard amounts. The endogenous level of analyte in the matrix was determined beforehand (abundance run) and served as the constant parameter [17,30]. For all biomarker candidates, the calibration curves obtained for the different peptides monitored were linear over the concentration ranges tested, and correlation coefficients were excellent (Fig. 2, Table 1). For NGAL and L-FABP, the quantification results for the different peptides were found to be very consistent. The accuracy (trueness) of the calibration curves was excellent for MIOX, NGAL and L-FABP, ranging between 93% and 97%. For PCK1, the LTPIGYIPK peptide provided measurements with 97% accuracy. The four additional signature peptides provided quantification values above 120%. This overestimation might be due to the instability of PCK1 proteolytic fragments which was already described by Ballard and coworkers [31]. As unlabeled recombinant PCK1 and its PSAQ standard have slight structural differences (Supplementary Fig. 2), the reduction of urea concentration from 4 M to 1 M during the MED-FASP protocol might have triggered differential precipitation of PCK1 proteolytic fragments. Regarding analytical precision, 10 of the 11 tracked signature peptides were associated with a CV below 15%, thus conforming to the most exacting recommendations made by health authorities and the proteomics community [17]. Based on the 7 signature peptides providing quantification accuracy between 80% and 120%, LLOQ could be determined according to the FDA definition and was below the ng/mL of urine for MIOX, PCK1 and L-FABP. For NGAL, the LLOQ was determined to be 2.4 ng/mL of urine. The analytical performances, including LLOQ values of the multiplexed proteomic assay are presented in Table 1. In summary, the proteomic assay displayed excellent analytical performances and was therefore suitable for simultaneously measuring the urinary concentration of the four biomarker candidates from an initial volume of just 400 µL.

3.4. Quantification of AKI biomarker candidates in urine samples

Urine samples (400 µL each) from healthy donors (n=10) and AKI patients with tubular (n=7) or glomerular injury (n=7) were spiked with defined amounts of PSAQ standards and prepared according to the MED-FASP protocol (Fig. 1). The 24 digested samples were then analyzed by LC-SRM in a randomized order as previously described. MIOX was detected in 8 out of the 10 urine samples obtained from healthy donors and was quantified in 6 samples at a mean concentration of 2.6 ± 1.4 ng/mL. Due to weak signals for its endogenous NYTSGPLLDR peptide, MIOX was not detected in most urinary samples from AKI patients. MIOX was quantified in only 4 out of the 14 samples tested (Table 2, Supplementary Fig. 4). Similarly, PCK1 was quantified in only 5 out of the 14 urine samples from AKI patients (Supplementary Fig. 4). In contrast, NGAL was quantified in most urine samples obtained from AKI patients (11 out of 14 urine samples) and in 6 out of 10 samples from healthy donors. As expected, urinary levels of NGAL were significantly higher in AKI patients than in healthy donors (Fig. 3A). However, the levels of this protein did not discriminate between patients with tubular versus glomerular injury (Fig. 3B). Finally, L-FABP was quantified in all urinary samples based on the

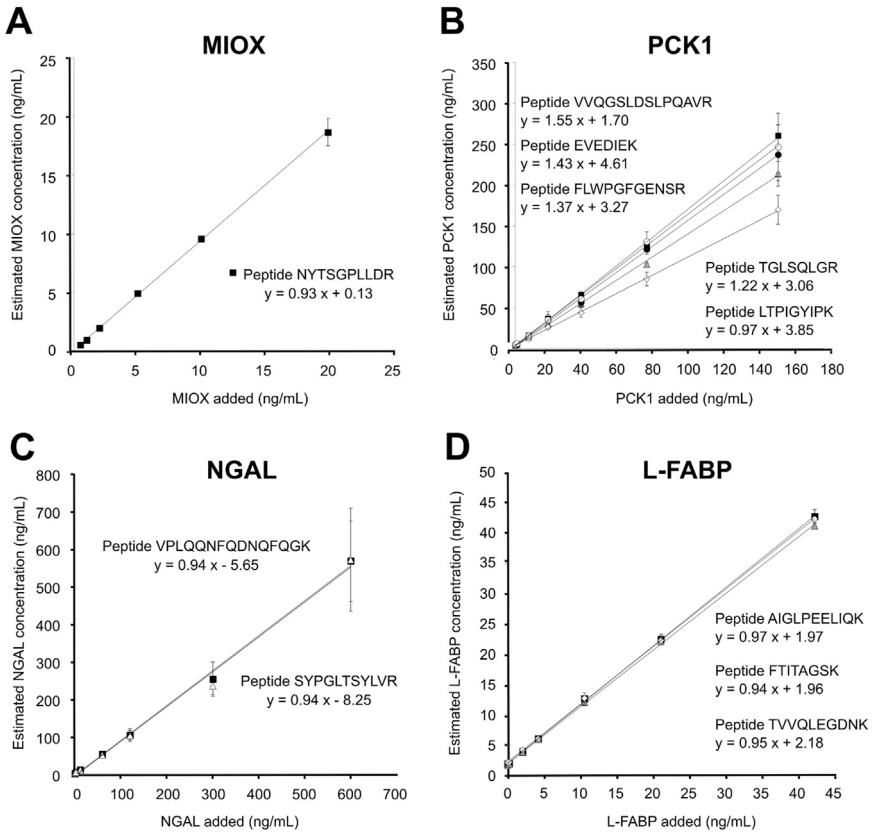

Fig. 2. Calibration curves obtained for AKI biomarker candidates. Calibration curves obtained for MIOX (**A**), PCK1 (**B**), NGAL (**C**) and L-FABP (**D**). Detailed information about the design of these calibration curves can be found in the Material and Methods section.

Table 1
Analytical performance characteristics of the proteomic assay.

Biomarker candidate	Peptide monitored	Range of concentrations tested (ng/mL of urine)	Linearity (R^2)	Accuracy (trueness)[a] (%)	LLOQ[b] (ng/mL of urine)	Precision at LLOQ (CV in %)
MIOX	NYTSGPLLDR	0.5 – 20.0	0.99	93	0.5	7
PCK1	VVQGSLDSLPQAVR	0.9 – 170.7	0.99	155	ND	ND
	TGLSQLGR	0.9 – 170.7	0.99	122	ND	ND
	FLWPGFGENSR	0.9 – 170.7	0.99	137	ND	ND
	LTPIGYIPK	0.9 – 170.7	0.99	97	0.9	10
	EVEDIEK	0.9 – 170.7	0.99	143	ND	ND
NGAL	VPLQQNFQDNQFQGK	2.4 – 598.3	0.99	94	2.4	6
	SYPGLTSYLVR	2.4 – 598.3	0.99	94	2.4	6
L-FABP	AIGLPEELIQK	0.2 – 42.3	0.99	97	0.2	6
	FTITAGSK	0.2 – 42.3	0.99	94	0.2	5
	TVVQLEGDNK	0.2 – 42.3	0.99	95	0.2	7

[a] Trueness corresponds to the slope value (%) of the calibration curve for the peptide considered.
[b] LLOQ was defined according to the FDA guidelines for bioanalytical method validation.

analysis of three signature peptides. Interestingly, the signals obtained for these signature peptides were unaffected by the increase in urine protein complexity in AKI patient urine. Statistical analysis indicated that the increase in L-FABP urinary concentration seen in AKI patients compared to healthy donors was significant (Fig. 3C). However, this protein could not distinguish between the two AKI patient groups (tubular vs. glomerular injury) (Fig. 3D). In summary, NGAL and L-FABP appear to be valuable biomarker candidates for diagnosis of AKI. In our small cohort, NGAL and L-FABP urinary levels could not differentiate tubular from glomerular injury.

4. Discussion

Due to its multiplexing capabilities, targeted proteomic analysis has the potential to solve the technological hurdle of biomarker evaluation. However, application of targeted proteomics as part of biomarker development requires key analytical performances to be attained, including specificity, sensitivity and confident quantification [6]. The goal of this study was to develop and assess a targeted proteomic pipeline to simultaneously evaluate 4 AKI biomarker candidates in urine samples. Thanks to a generic and efficient sample preparation method (MED-FASP) and the use of PSAQ standards for quantification, our multiplexed proteomic assay demonstrated excellent analytical performance, in line with recommendations from the health

Table 2
Quantification of biomarker candidates in urine samples.

Patient number	Disease	Urinary creatinine (mmol/l)	Candidate biomarker concentrations determined by LC-SRM (ng/mL of urine)				Candidate biomarker concentrations expressed relative to urinary creatinine levels (mg/mol of creatinine)[b]			
			MIOX	PCK1[a]	NGAL	L-FABP	MIOX	PCK1	NGAL	L-FABP
1	Tubular AKI	22.7	ND	ND	ND	137.7	ND	ND	ND	6.1
2		1.8	ND	ND	ND	146.7	ND	ND	ND	81.5
3		1.8	ND	ND	1188.5	73.8	ND	ND	660.3	41.0
4		10.9	ND	16.2	483.0	67.8	ND	1.5	44.3	6.2
5		5.4	1.2	4.2	153.5	59.1	0.2	0.8	28.4	10.9
6		7.1	2.1	ND	156.5	10.2	0.3	ND	22.0	1.4
7		4.7	ND	9.6	105.5	19.2	ND	2.0	22.4	4.1
8	Glomerular AKI	8.5	ND	ND	62.5	86.4	ND	ND	7.4	10.2
9		7.7	1.1	ND	26.0	123.9	0.1	ND	3.4	16.1
10		7.1	ND	ND	92.5	65.4	ND	ND	13.0	9.2
11		9.4	8.0	20.7	ND	23.1	0.9	2.2	ND	2.5
12		2.8	ND	ND	192.0	47.7	ND	ND	68.6	17.0
13		2.2	ND	14.1	172.5	115.2	ND	6.4	78.4	52.4
14		13.0	ND	ND	53.5	39.9	ND	ND	4.1	3.1
15	Healthy donors	14.5	1.2	5.4	8.5	1.8	0.1	0.4	0.6	0.1
16		20.9	4.8	28.8	16.0	9.6	0.2	1.4	0.8	0.5
17		3.1	< LLOQ	< LLOQ	ND	0.9	< LLOQ	< LLOQ	ND	0.3
18		11.9	3.6	19.2	193.0	6.0	0.3	1.6	16.2	0.5
19		8.5	1.7	6.6	42.0	5.1	0.2	0.8	4.9	0.6
20		11.4	1.4	6.6	30.5	6.6	0.1	0.6	2.7	0.6
21		1.1	ND	ND	ND	0.3	ND	ND	ND	0.3
22		5.4	2.7	10.8	20.5	6.3	0.5	2.0	3.8	1.2
23		1.4	ND	ND	ND	0.6	ND	ND	ND	0.4
24		4.8	< LLOQ	2.7	11.5	1.2	< LLOQ	0.6	2.4	0.3

ND: Not Determined.
[a] The five PCK1 signature peptides were considered to calculate PCK1 concentrations.
[b] Normalization relative to urinary creatinine concentration was used to correct for variations in urine dilution.

authorities and the proteomics community [17]. The major advantages of our assay are its multiplexing capabilities, its high specificity (due to monitoring of signature peptides), its high sensitivity (LLOQ < ng/mL of urine) and its quantification performance (accuracy, precision, linearity). These performance criteria are essential to deliver reliable analyte measurements and interpretable biological data. In addition, molecular interactions involving the targeted biomarkers were overcome by the denaturation and reduction steps performed before protein digestion and LC-SRM analysis. These interactions are a major source of variability in immunoassays, especially multiplexed assays.

In the field of nephrology, AKI is routinely diagnosed based on functional parameters, but improvements to patient care and therapeutic choices could be made if it were possible to determine the site and extent of nephron injury at early stages. Recently, two glomerular proteins (podocin and podocalyxin) and one tubular protein (MIOX) were identified as potential biomarkers of nephron injury [9,12,23]. Assays were developed based on the use of specific antibodies [23] or quantitative targeted proteomics [9,12] for their ongoing clinical evaluation. In line with these studies, we selected PCK1 as a potential AKI biomarker as it is expressed by the proximal tubular cells and may leak into urine following tubular injury [32]. Notably, PCK1 is also expressed in hepatocytes and may be present in the blood following liver injury. However, with a molecular weight of over 72 kDa, it is not expected to pass through the glomerular pores, and should therefore not be present in primary urine unless glomeruli are also injured. In this study, endogenous PCK1 was detected in very few urine samples (although PCK1 PSAQ standard generated detectable signature peptides). This result could be because PCK1 is very sensitive to urinary proteases and/or because it is an unstable protein [31]. The enzyme MIOX is also specifically expressed in the proximal tubule, which is why Gaut and coworkers selected it as a potential AKI biomarker [23]. Their results indicated increased serum levels in AKI patients at early stages, as a consequence of tubular back-leak. In urine, our results indicated barely detectable MIOX levels in AKI patients, whatever the site of nephron injury. In contrast, it could be detected in the urine of 8 out of 10 healthy donors. Thus, at the protein level, our results indicate that urinary MIOX might be used as a potential renal recovery biomarker rather than a marker of tubular injury. Overall, these results indicate that biomarker candidates of kidney injury should not be selected only based on biological criteria such as cell restricted expression. Their detectability in the matrix should also be taken into account at early stages of evaluation. Along this line, we noticed that NGAL and L-FABP were much more easily detected in urine than PCK1 and MIOX (Supplementary Fig. 4). This was possibly because of greater resistance to proteolytic degradation, NGAL being covalently linked to MMP9, and L-FABP interacting with small hydrophobic molecules [33]. These two proteins have already been the subjects of several studies for AKI diagnosis and have entered the last stages of biomarker development [1,34]. In our small AKI patient cohort we were able to confirm the clinical relevance of these two urinary proteins for AKI diagnosis. Interestingly, the panel of proteins monitored could readily be extended to other candidate biomarkers using stable isotope-labeled peptides or PSAQ standards. Thus, KIM-1 (Kidney Injury Molecule-1), IL-18 (interleukin 18) and cystatin-C, all of which have been proposed as candidate biomarkers for early detection of AKI [1,2], could be included in the test panel. These small, soluble proteins should be relatively easy to synthesize in a labeled recombinant form (PSAQ standard) [24].

5. Conclusion

In this study, we developed a targeted proteomic pipeline to accurately quantify four urinary proteins which are potential AKI biomarkers. Beyond the biological results, confirming the relevance

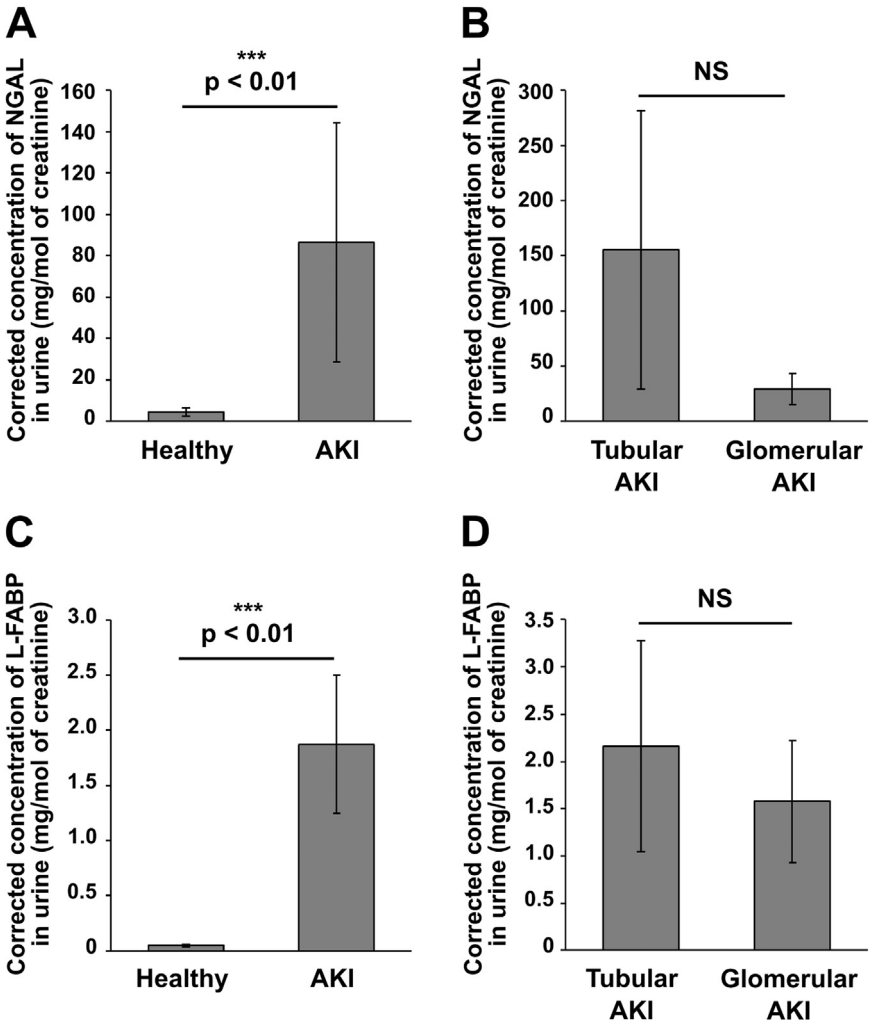

Fig. 3. Urinary NGAL and L-FABP levels are significantly elevated in AKI patients compared to healthy donors. Comparison of urinary NGAL and L-FABP levels between healthy donors and AKI patients (**A** and **C**), as determined by LC-SRM analysis. Comparison of urinary NGAL and L-FABP levels between AKI patients with tubular injury and those with glomerular injury (**B** and **D**). Biomarker concentration in urine was expressed relative to urinary creatinine levels to reduce the impact of urine dilution. Statistical significance was calculated using the Mann-Whitney-Wilcoxon test.

of NGAL and L-FABP in AKI diagnosis (as independent parameters or in combination), this proteomic assay constitutes the basis for further analytical developments and future clinical studies dedicated to the evaluation of multiple urinary protein biomarkers in a myriad of clinical contexts.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2016.11.023.

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