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Tandem mass spectrometry analysis of urinary podocalyxin and podocin in the investigation of podocyturia in women with preeclampsia and Fabry disease patients

Tristan Martineau[a], Michel Boutin[a], Anne-Marie Côté[b], Bruno Maranda[a], Daniel G. Bichet[c], Christiane Auray-Blais[a,*]

[a] Division of Medical Genetics, Department of Pediatrics, Université de Sherbrooke, Centre de recherche-CHUS, 3001, 12th Avenue North, Sherbrooke, Quebec J1H 5N4, Canada
[b] Division oef Nephrology, Department of Medicine, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Centre de recherche-CHUS, 3001, 12th Avenue North, Sherbrooke, Quebec J1H 5N4, Canada
[c] Sacré-Coeur Hospital, Université de Montréal, Quebec, Canada

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ABSTRACT

Background: Podocytes are highly differentiated visceral cells, and several related specific proteins, such as podocalyxin and podocin are potential tools for the evaluation of podocyturia. However, precise quantitation of podocyturia-related proteins is complex and often unreliable.
Method: A reversed-phase ultra-performance liquid chromatography coupled to tandem mass spectrometry method was developed and validated to quantify podocalyxin and podocin levels in urine supernatant by using specific cleavable peptides and standards. Urine samples from women with normotensive or hypertensive pregnancies, gestational diabetes and preeclampsia, as well as treated and untreated Fabry patients, and gender-matched controls were investigated.
Results: The multiplex analysis shows that podocalyxin levels were higher than podocin levels in patients, the former being particularly higher in pregnant women. Women with preeclampsia had abnormal urine levels of both proteins with a higher sensitivity for podocalyxin. Slightly increased levels of podocin were also observed in Fabry males, while both proteins were increased in untreated Fabry females. Correlations were established between podocalyxin and podocin levels and clinical parameters associated with Fabry disease and pre-eclampsia.
Conclusions: This methodology makes possible the precise, simultaneous and reliable analysis of podocalyxin and podocin levels, and offers a valuable tool for the evaluation of podocyturia.

1. Introduction

Chronic kidney disease (CKD) is associated with biochemical disturbances contributing to multi-morbidity, kidney failure and a requirement for dialysis or kidney transplantation [1]. The worldwide prevalence of CKD is estimated at 13% and it is recognized as a major contributor to the costs of healthcare [2]. The measurements of albuminuria-creatinine ratio (ACR) and estimated glomerular filtration rate (eGFR) are currently the most widely used predictors of CKD prognosis and kidney involvement [3]. Unfortunately, the use of these conventional tools are not always reliable in the case of the chronic kidney disease occurring in Fabry disease and in preeclampsia [4,5].

Fabry disease is a rare, multisystemic X-linked lysosomal storage disorder (OMIM #301500) caused by mutations in the *GLA* gene (Xq22.1) encoding the enzyme alpha-galactosidase A (α-gal, EC 3.2.1.22) which leads to the impaired catabolism of glycosphingolipids. To date, over 900 mutations in the *GLA* gene are reported leading to Fabry disease [6,7]. The accumulation of globotriaosylceramide (Gb_3), globotriaosylsphingosine (lyso-Gb_3) and galabiosylceramide (Ga_2) in the vascular endothelium, different organs, tissues and biological fluids is one of the main biochemical features of Fabry patients. Progressive kidney failure is one of the principal clinical complications of the disease [8,9]. Enzyme replacement therapy (ERT) by infusion of two accepted medications, such as agalsidase beta (1 mg/kg, Fabrazyme, Genzyme-Sanofi) or agalsidase alfa (0.2 mg/kg, Replagal, Shire Human Genetic Therapies) has been demonstrated to delay disease progression.

* Corresponding author.
E-mail address: christiane.auray-blais@usherbrooke.ca (C. Auray-Blais).

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Recently, chaperone therapy, a new promising oral treatment, was developed to treat Fabry patients with residual enzyme activity who have amenable mutations [10]. Treatment guidelines depend on mutation types (classical or late-onset), gender and specific clinical manifestations based on organ involvement [11]. However, the diagnosis of Fabry patients is often delayed (sometimes by > 15 years) owing to the marked variability in the phenotype and genotype, particularly in late-onset patients having no early signs of the disease [11,12].

Preeclampsia is a gestational hypertensive disorder affecting 3–5% of pregnancies and remains one of the major causes of maternal and fetal mortality and morbidity around the world [13]. The main diagnostic criteria are: 1) hypertension after 20 gestational weeks (systolic pressure ≥ 140 mmHg or diastolic pressure ≥ 90 mmHg); and 2) proteinuria (urinary protein:creatinine ratio ≥ 30 mg/mmoL); or 3) evidence of other hepatic, neurological, haematological or uteroplacental signs/dysfunction. The most effective treatment is maternal delivery [13,14]. Unfortunately, clinical manifestations in women with preeclampsia are quite variable, or usually appearing late in pregnancy, leading to a delay in the management of affected women [13]. A rise in urinary podocyte levels in preeclampsia patients was observed in previous studies compared to controls [15–17].

Several studies have shown that the evaluation of podocyturia may be an effective biochemical indicator of kidney damage [18–20]. Podocytes are highly differentiated glomerular cells with limited capacity for cell division that form the interdigitating foot processes located on the outer surface of the glomerular capillaries in the Bowman's capsule [21]. Podocytes are essential for the permeability of the capillary filtration barrier and limit the passage of macromolecules, such as albumin, to the glomerular filtrate. The detachment of podocytes caused by membrane damage may lead to effacement of the foot processes, proteinuria and progressive kidney failure. Several unique proteins such as podocalyxin, podocin, nephrin and synaptopodin are located within these cells or in the extracellular vesicles [21–23]. Effacement of foot processes and increased concentrations of podocytes in the urine in patients with Fabry disease has been shown to correlate with clinical severity of Fabry nephropathy, and it has been recommended that it be used as a tool to evaluate kidney involvement for the initiation of ERT in some cases [10,23,24].

Current methodologies for the evaluation of podocyturia in patients are challenging. [24–27]. Methods employing the immunofluorescence quantitation of specific proteins, such as by ELISA, and cell counting [22–26], to evaluate podocyturia are time-consuming, offer a limited sensitivity, and are expensive owing to the need to use specific antibodies [25,26]. The quantitative analysis of mRNAs by a quantitative polymerase-chain reaction tool (qPCR) has been used to quantify specific podocyte protein mRNAs in urine [16,28,29], but the quality of mRNAs is altered by bacteria contamination leading to increased variability [25,30]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods were also developed to evaluate podocyturia using protocols to quantify urinary podocalyxin and podocin separately [31–33]. The quantitation strategy was based on the absolute protein quantitation using stable isotope labelled peptides (absolute quantitation (AQUA)) [34]. However, in a recent study, it was shown that the AQUA technique using stable-isotope-labelling peptides without cleavable sequences may lead to an underestimation of the protein abundance owing to incomplete proteolysis during the sample preparation [35,36].

A methodology based on LC-MS/MS technology offers major benefits for clinical analysis, such as good sensitivity, high specificity, reduced sample volume for analyses, and the possibility of simultaneous protein/peptide analysis, and it is amenable to protein quantitation. Mass spectrometry is a powerful tool for protein quantitation based on the analysis of ions obtained from peptide fragments characteristic of the targeted proteins by proteolytic digestion often using trypsin, which hydrolyzes peptide bonds on the C-terminus side of arginine or lysine residues when not followed by a proline residue [37].

The main objective of this research project was to develop and validate an efficient and robust ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the simultaneous quantitation of podocalyxin and podocin in urine specimens. This objective was based on a strategy involving a cleavable isotope-labelling peptide to evaluate specific podocyte protein levels in urine. The secondary aim focused on the analysis of podocalyxin and podocin levels in Fabry patients, women with preeclampsia and related gender-matched controls. Overall, this proposed methodology provides evidence supporting a technological transfer to the clinical field for monitoring kidney involvement.

2. Material and methods

2.1. Ethics approval

This multicenter project was approved by the Research Ethics Board (REB) of the Faculty of Medicine and Health Sciences and the Centre intégré universitaire de santé et de services sociaux de l'Estrie – Centre hospitalier universitaire de Sherbrooke (CIUSSS de l'Estrie - CHUS) and the Hôpital du Sacré-Coeur in Montreal, Quebec, respecting the Declaration of Helsinki. Informed consent was obtained from all Fabry patients, women with preeclampsia and healthy controls.

2.2. Patients and controls selected

Random urine specimens (50 ml) were collected from healthy controls, pathological controls, women with preeclampsia and Fabry patients after obtaining their informed consent. Inclusion criteria were selected according to specific clinical manifestations as follows: all Fabry patients had been previously diagnosed according to the Canadian Fabry Disease Initiative (CFDI) guidelines [38]. Female patients treated by ERT (TFF; $n = 6$) and males with Fabry on ERT (TFM; $n = 11$) were either receiving agalsidase-alfa or agalsidase-beta according to the dosage previously described. Untreated Fabry females (UFF; $n = 10$) and untreated Fabry males (UFM; $n = 2$) were recruited from among patients who were not eligible for ERT according to the CFDI guidelines. Women with preeclampsia (PE; $n = 13$) were identified by the occurrence of gestational hypertension arising de novo at or after 20 weeks (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic) combined with proteinuria (≥ 30 mg of protein/mmol of creatinine). Women with gestational diabetes (GD; $n = 9$) and pregnant women with hypertension, but not preeclampsia, (HP; $n = 8$) were included as pathological controls because of their potential kidney involvement [39,40]. The diagnosis of gestational diabetes was made according to the Canadian Clinical Practice Guidelines employing the 50-g glucose tolerance test [41]. Pregnant women with hypertension were identified by a rising blood pressure de novo (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic) without other signs or symptoms at or after 20 weeks of gestation. The severity of the hypertension (according to the recommendations of the Canadian guidelines) for this latter group was not taking into account because the number of eligible pregnancies was low [42]. Healthy control males (CM; $n = 10$) and females (CF; $n = 10$) did not have Fabry disease or any other lysosomal storage disorders or nephropathy abnormalities. Normotensive pregnant women had normal blood pressure, and did not have diabetes, nephropathy or other complications of pregnancy. However, patients treated by dialysis or kidney transplant and all participants under 18 years old were excluded from this study. Clinical manifestations other than the ones selected in the inclusion criteria were excluded from this study.

2.3. Materials

2.3.1. Reagents and solvents

Optima LC/MS grade water, isopropyl alcohol (IPA), A.C.S. reagent

grade ammonium formate (NH_4HCO_2, Amm. Form.), and ammonium bicarbonate (NH_4HCO_3) were from Fisher Scientific. Formic acid (FA) (> 99%) was from Acros Organics. HPLC grade methanol (MeOH) and acetonitrile (ACN) were from EMD Chemicals Inc. Dithiothreitol (DTT) (> 98%), iodoacetamide (IAA), sodium deoxycholate (DOC) (> 97%), porcine trypsin IX-S with 13,000–20,000 BAEE units/mg were obtained from Millipore Sigma. Oasis™ mixed-mode strong cation-exchange (MCX) cartridges (3 cc, 60 mg) were from Waters Corp. Synthetic human urine (Surine) was from BioIVT. All buffers were freshly prepared.

2.3.2. Recombinant proteins and synthesized peptide standards

Recombinant podocin (NPHS2, > 80%) was from Novus Biologicals while recombinant podocalyxin isoform 2 (PODXL, > 80%) was from Creative BioMart. JPT Peptide Technologies Gmbh synthesized the stable isotope-labelled (SIL) peptides (APAATVVDVDEVR*; ATFNPAQDK*), the cleavable stable isotope-labelled (CSIL) peptides (CRAVK/ATFNPAQDK*/CGIRL; PGEPR/APAATVVDVDEVR*/GSGEE) and their unlabelled counterparts (APAATVVDVDEVR; ATFNPAQDK; CRAVK/ATFNPAQDK/CGIRL; PGEPR/APAATVVDVDEVR/GSGEE). Heavy arginine residues (R*) were labelled with $^{13}C_6$ and $^{15}N_4$ (+10 Da) while heavy lysine residues (K*) were labelled with $^{13}C_6$ and $^{15}N_2$ (+8 Da). All peptides were quantified by the manufacturing company for quality assessment using an amino acid analysis (AAA) (> 98%).

2.4. Sample preparation for the analysis of podocalyxin and podocin

2.4.1. Urine sample specimens

Fresh random urine specimens (50 ml) were collected, homogenized and divided in half: one 25-ml aliquot was centrifuged at 2000 ×*g* for 15 min and the other 25-ml aliquot was saved uncentrifuged. The supernatant of the centrifuged urine samples (the sediments were discarded) and the uncentrifuged specimens were stored at −80 °C until analysis. Creatinine concentration, albuminuria and proteinuria levels were measured in the uncentrifuged samples with the automated Modular P system from Roche at the Biochemical Clinical Laboratory at CIUSSS de l'Estrie-CHUS. Total urinary Gb_3 on filter paper and lyso-Gb_3 in urine specimens were analyzed in our laboratory according to published methods [44,43].

One milliliter of urine supernatant was transferred to a 2 ml polypropylene tube with a screw cap along with 20 µl of 100 nmol/l of podocalyxin and podocin CSIL as the internal standards. A volume of 100 µl of 7 mg/ml DOC prepared in 550 mmol/l NH_4HCO_3 (pH 8), followed by 100 µl 225 mmol/l DTT prepared in 50 mmol/l NH_4HCO_3 were added to the samples, followed by a 30 min incubation at 60 °C (Incubating Orbital Shaker, VWR, operated at 255 rpm) to reduce the disulfide bonds. After adding 250 µl of 180 mmol/l IAA prepared in 50 mmol/l NH_4HCO_3, samples were incubated for 30 min in the dark at room temperature (23 °C) to alkylate the cysteine residues. Enzymatic digestion was performed with 50 µg porcine trypsin IX-S and incubated 2 h at 37 °C with orbital shaking (225 rpm). The reaction was quenched with 30 µL 25% F.A. and then centrifuged at 9400 ×*g* for 10 min to eliminate the DOC precipitate before the sample loading on a solid phase extraction OASIS™ MCX cartridge (3 cc, 60 mg) previously conditioned with 1 ml MeOH and 1 ml H_2O containing 0.5% F.A. Cartridges were washed consecutively with 1 ml 70:30 H_2O:MeOH containing 0.5% F.A., 1 ml H_2O containing 0.5% F.A., 1 ml 200 mmol/l Amm. Form., and eluted in a 5 ml polypropylene tube with 1 ml 80:20 (100 mmol/l Amm. Form.:ACN). Extracted samples were evaporated for 3 h at 45 °C with a SpeedVac (SPD131DDA ThermoFisher Scientific) at 1 Torr and reconstituted in 100 µl 85:15 H_2O:ACN containing 0.5% F.A. before filtration on a 0.2 µm PTFE syringe filter. Analyses were done on an Acquity I-Class ultra-performance liquid chromatography system (UPLC, Waters) coupled to a Xevo TQ-S tandem mass spectrometer (MS/MS, Waters).

2.4.2. Calibration curves

Peptide calibration point solutions were prepared with cleavable peptides of podocalyxin and podocin in 50:50 MeOH:H_2O containing 0.1% F.A. at the following concentrations: 6.25; 12.5; 25; 50; 100; 200; 300 and 400 nmol/l. To avoid interferences of residual podocyte proteins in the calibration curve, 1 ml of urine was replaced by a synthetic human urine (Surine) combined to 20 µl of the respective peptide calibration point solutions. A final 9 point-calibration curve for the proteins of interest was obtained: 0; 125; 250; 500; 1000; 2000; 4000; 6000 and 8000 pmol/l.

2.5. Instrumentation and parameters

The analysis of tryptic peptides from the respective recombinant proteins was performed in order to select the appropriate peptide for the quantitation of podocalyxin and podocin. The separation of peptides was done on the Acquity I-Class UPLC system with a linear gradient reversed-phase UPLC method to reduce the matrix effect. The UPLC system was coupled to a Xevo TQ-S mass spectrometer working in positive electrospray ionization (ESI+) using the multiple reaction monitoring (MRM) mode. To determine the appropriate fragment ions used for the MRM analysis, produced y-ions in the MS/MS collision cell were analyzed and compared with Skyline 4.2 software (MacCoss Lab Software). Optimal UPLC-MS/MS parameters of the selected podocalyxin and podocin peptides are shown in Table 1. The method was validated and the MRM results were obtained using the MassLynx-TargetLynx V4.1 (SCN 945) software (Waters). The quadratic calibration curves were forced to the origin and a 1/x weighing was applied.

2.6. Method validation

The method validation was performed for research purposes. Spiked urine quality controls (U-QCs) and spiked synthetic urine quality controls (S-QCs), with selected cleavable peptides of podocin and podocalyxin, were prepared at final concentrations of 0.3 nmol/l (LQC), 2.5 nmol/l (MQC) and 5.0 nmol/l (HQC). All QCs were individually prepared in 2 ml polypropylene tubes aiming at a final volume of 1 ml. The S-QCs were used to evaluate assays for intraday (n = 5) and interday (n = 5) accuracy in order to prevent endogenous podocyte proteins to interfere with the accuracy evaluation, while U-QCs were used to evaluate precision. U-LQCs and U-HQCs were used to evaluate the sample stability at different times and temperatures: 24 h at room temperature (22 °C) (n = 3), 1 week at 4 °C (n = 3), 2 weeks at –20 °C (n = 3), and 2 weeks at –80 °C (n = 3). The effect of 5 freeze-thaw cycles (n = 3) was also assessed for both proteins. The stability of the reconstituted U-QCs was also evaluated up to 48 h in the autosampler at 20 °C (n = 5) and their adsorption to polypropylene tubes was evaluated after 5 transfers (n = 3). The limit of detection (LOD) and the limit of quantitation (LOQ) were established as 3 and 10 times the standard deviations of the analyte response, respectively, which was obtained after 5 injections of S-LQC. The overall sample preparation recovery was evaluated by comparing U-LQC and U-HQC containing cleavable peptides at concentrations initially known and the equivalent matrix containing peptides without cleavable ends, spiked after the evaporation step (n = 5). The matrix effects (suppression or enhancement of ion signals) were evaluated using a post-column infusion strategy. Urine samples with different creatinine concentrations (3.4 to 18.6 mmol/l) and total protein concentrations (0 to 1.05 mg/ml), and the reconstitution solution (85:15 H_2O:ACN containing 0.5% F.A) were analyzed by UPLC separation to evaluate different matrix compositions. At the same time, the signal of the SIL peptides infused at a concentration of 10 nmol/l with a flow rate of 20 µl/min was monitored to measure ion suppression or enhancement at the retention times of the analytes.

Table 1
UPLC and MS/MS method parameters for the analysis of urine specific peptides (at equivalent molar concentrations) for podocalyxin and podocin.

Chromatography paramaters			MS parameters		
Column	CORTECS T3 (Waters Corps.)		Ionization mode	ESI +	
ID x length	2.1 × 50 mm		Acquision mode	MRM	
Particle size	1.6 µm		Capillary voltage	3.0 kV	
Temperature	35 °C		Nebuliser gas flow	6.0 Bar	
Flow rate	0.6 ml/min		Cone gas flow	500 l/h	
Injection volume	7.5 µl (partial loop)		Source offset voltage	90 V	
Mobile phase A	100% ACN + 0.1% F.A.		Desolvation temperature	600 °C	
Mobile phase B	100% H_2O + 0.1% F.A.		Desolvation gas flow	1200 l/h	
Weak wash	3000 µl 95% H_2O/5% ACN + 0.1% FA		Collision gas flow	0.15 ml/min	
Strong wash	1000 µl 60% ACN/30% IPA/10% H_2O + 0.2% FA		Source temperature	150 °C	
Time (min)	Mobile phase %	Gradient type	MRM parameters	Podocalyxin	Podocin
0.0–1.5	5% A	Isocratic	Precursor ion selected – y	$[ATFNPAQDK + 2H]^{2+}$- (y_5)	$[APAATVVDVDEVR + 2H]^{2+}$- ($y_7$)
1.5–6.0	5–17% A	Linear	MRM transition – peptide	*m/z* 496.25 > 558.29	*m/z* 671.35 > 831.42
6.0–7.0	17–100% A	Linear	MRM transition – IS	*m/z* 500.25 > 566.29	*m/z* 676.35 > 841.42
7.0–8.0	100% A	Isocratic	Cone voltage	20 V	30 V
8.0–9.0	100–5% A	Linear	Collision energy	15 V	26 V
9.0–10.0	5% A	Isocratic	Dwell time	0.12 s	0.06 s

IS: internal standard; ACN: acetonitrile; IPA: isopropyl alcohol; H_2O: water; FA: formic acid; MS: mass spectrometry; MRM: multiple reaction monitoring; UPLC: ultra-performance liquid chromatography; ID: internal diameter.

2.7. Statistical analyses

IBM SPSS Statistics 24 software (SPSS, Armonk, New York, USA) was used for all statistical analyses. Comparisons between different biomarker levels from pathological controls, patients and healthy controls were performed using the non-parametric Mann–Whitney *U* test. Normal values for podocalyxin and podocin were established gender-wise with a 95th percentile measurement of biomarker levels in urine from the respective healthy controls, and the pregnancy status for women with preeclampsia. Reference values were used to calculate the sensitivity (true positive/(true positive + false negative)) and the specificity (true negative/(true negative + false positive)) and to determine the area under the curve (AUC) of receiver operating characteristic (ROC) to evaluate the diagnostic reliability of podocalyxin and podocin as biomarkers of podocyturia. Correlations between podocyturia proteins and clinical manifestations were evaluated with the non-parametric Spearman test.

3. Results

3.1. Selection of the podocalyxin and podocin peptide sequences for UPLC-MS/MS analysis

Selection of the appropriate peptide sequences for the protein quantitation by MS/MS in MRM mode was crucial to ensure the reproducibility, robustness and highest sensitivity of the methodology. Briefly, we selected tryptic peptide sequences with a minimum of 8 amino acids for specificity and a maximum of 25 amino acids for sensitivity. We also favoured sequences with the minimum number of residues subject to phosphorylation such as serine, threonine or tyrosine to ensure reproducibility of the assay [30]. As mentioned previously, recombinant proteins of podocalyxin and podocin were digested with trypsin and were analyzed as described in the Supplementary information methodology 1 and 2. Fig. 1A shows three proteolytic peptides producing the highest signals for podocalyxin (ATFNPAQDK, ANEILASVK and LASVPGSQTVVVK), whereas Fig. 1C shows the ones related to podocin (APAATVVDVDEVR, MAAEILSGTPAAVQLR and QEAGPEPSGSGR). The 3 most abundant fragment ions were analyzed to evaluate the most sensitive y-ion produced by MS/MS from the selected peptides. Fig. 1B and D show the respective y-ion profiles generated from the doubly charged precursor ions for both analyzed peptides. The singly charged fragment y_5 ion (*m/z* 558.29) from the ATFNPAQDK doubly charged precursor ion (*m/z* 496.25) and the singly charged fragment y_7 ion (*m/z* 831.42) from the APAATVVDVDEVR doubly charged precursor ion (*m/z* 671.35) were selected for the MRM transitions (Table 1). Although they were all unique, ATFNPAQDK and APAATVVDVDEVR had been conserved for the development of the quantitative method because of their sensitivity endogenously and their limited number of post-translational modifications compared to other peptide sequences. MS/MS methods previously developed for the individual quantitation of podocalyxin and podocin using the AQUA quantification strategy selected the same peptides [32,33]. One major limitation of these methods is that the use of internal standards without cleavable sequences could underestimate the endogenous protein concentrations in urine because of incomplete trypsin digestion. The use of stable isotopic labelled proteins as internal standards was suggested as the most appropriate approach to resolve this issue [36]. However, these protein standards are difficult to produce and quite expensive. Hence, as a compromise, cleavable stable isotope-labelled (CSIL) peptides and their native sequences were synthesized, adding 5 amino acids to the original protein sequences to the N-terminus and the C-terminus to our selected peptides (Podocalyxin: CRAVK/ATFNPAQDK/CGIRL, and podocin: PGEPR/APAATVVDVDEVR/GSGEE) to compensate for missed-cleavages during the tryptic digestion.

3.2. Method validation results

LOD, LOQ, intra- and interday accuracy and precision assays for podocalyxin and podocin are summarized in Supplementary material (Table S-1), while recovery of sample preparation and polypropylene adhesion assays are summarized in Supplementary material (Table S-2), and all stability results are shown in Supplementary material (Table S-3). LOD measured for podocalyxin and podocin target peptides were 17.1 and 19.0 pmol/l, respectively. The LOQ obtained was 56.9 and 63.3 pmol/l, respectively. Precision (% RSD, Relative Standard Deviation) assays were ≤ 6.5% and accuracy assays (% bias) were ≤ 13.9% for both peptides of podocalyxin and podocin at different concentrations, except for the S-LQC intraday of podocalyxin and S-LQC and S-HQC intraday of podocin bias assays which were 15.9%, 16.4% and 17.4%, respectively, because of an outlier in the 5 replicates. By excluding the outlier, biases were reduced under 15%. Overall recoveries of sample preparation were between 65.2 and 69.8%, respectively, for LQC and HQC for the podocalyxin peptide, while they were between 72.9 and 81.2% for the podocin peptide. The maximum bias obtained for the adhesion to polypropylene tube assays after 5 transfers was under 2.5% for podocalyxin and podocin peptides. Cleavable targeted peptides were stable for at least 24 h at 22 °C (biases < 15.5%), at one week at 4 °C (biases ≤ 13.1%), at least 2 weeks at –20 °C (biases ≤

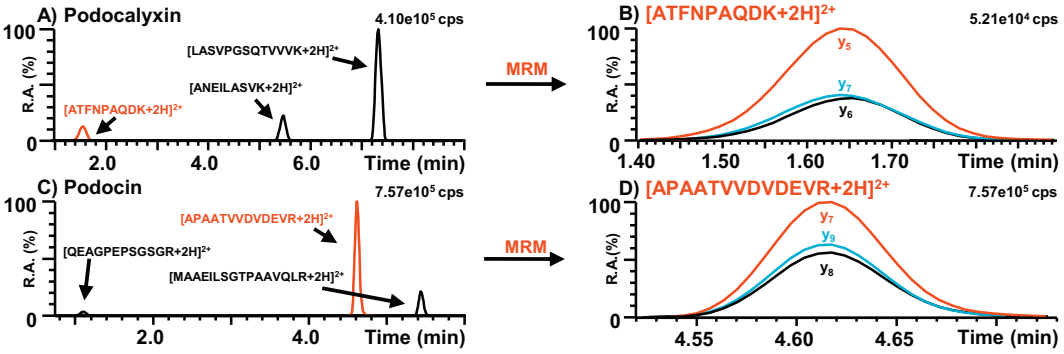

Fig. 1. Selection of specific peptides for the MS/MS quantitation of podocalyxin and podocin in MRM mode. A) Ion chromatogram of three tryptic peptides from recombinant podocalyxin protein (59.2 nmol/l) with their highest y-ion signal; B) Overlay of the ion chromatograms created by the MRM of 3 y-ions produced by the fragmentation of $[ATFNPAQDK + 2H]^{2+}$ precursor ion; C) Ion chromatogram of three tryptic peptides from the recombinant podocin protein (21.3 nmol/l) with their highest y-ion signal; D) Overlay of the ion chromatograms created by the MRM of three y-ions produced by the fragmentation of $[APAATVVDVDEVR + 2H]^{2+}$ precursor ion. R.A.: Relative abundance (%).

10.7%), and –80 °C (biases ≤ 11.8%) and even after 5 freeze-thaw cycles (biases ≤ 4.1%). Extracted LQC and HQC were stable at least 48 h in the autosampler at 20 °C (biases ≤ 6.0%).

The matrix effect was investigated to monitor the ion suppression on targeted peptides during MS/MS quantitation with three different urine sample matrices with creatinine levels varying between 3.4 and 18 mmol/l, and total protein levels between 0 and 1.05 mg/ml. The reconstitution solution (85% H_2O; 15% ACN; 0.5% F.A.) was defined as showing no ion suppression and was compared to the other matrices. As shown in Fig. 2, the ion suppression in a urine matrix without proteinuria was under 25.7%, but was greater in samples with higher creatinine concentration, especially for the targeted peptide of podocin. However, the impact of ion suppression was remarkably greater in urine with proteinuria especially for ATFNPAQDK (−40.0%) compared with APAATVVDVDEVR (−25.7%). An assay with the quadrupole time of flight mass spectrometer (QTOF) confirmed that the increased ion suppression was caused by the coelution of some tryptic peptides of human serum albumin which is the one of the most abundant proteins found in urine [45]. This latter assay is described in the Supplementary material, and results are summarized in Fig. S-1 and S-2.

3.3. Podocalyxin and podocin levels in random urine samples

The groups under study were subdivided according to pathological criteria, gender and treatment (Fabry patients only). For the Fabry disease groups, the levels of podocalyxin and podocin were compared to gender-matched controls. In the preeclampsia groups, podocalyxin and podocin levels were compared with healthy non-pregnant women (CF) and women with normotensive pregnancies (NP). Women with gestational diabetes (GD) and hypertensive pregnancies (HP) were used as pathological controls. Age, gender, eGFR measurements, diastolic blood pressure (DBP), systolic blood pressure (SBP), gestational age, age at delivery and α-gal activity in plasma are summarized in Table 2. Levels of podocalyxin, podocin, proteinuria, albuminuria, total Gb_3 on filter paper and lyso-Gb_3 in urine specimens normalized to creatinine are shown in Table 3. Total Gb_3 and lyso-Gb_3 were measured in healthy controls (without pregnancy) and in Fabry patients.

Fig. 3 shows the box plot comparisons of podocalyxin and podocin levels in different study groups.

Our results show that the proteinuria, albuminuria and blood pressure levels measured for each group matched the clinical manifestations of patients; statistically significant differences ($p < .05$) were assessed with the use of the non-parametric Mann Whitney *U* test (Supplementary material Table S-4). As expected, proteinuria, albuminuria, total Gb_3 and lyso-Gb_3 concentrations varied between ERT-treated and untreated Fabry groups. Fabry disease is an X-linked disorder where females tend in general to present lower levels of glycosphingolipid than males [46]. Fabry patients with classical *GLA* mutations also present higher biomarker concentrations than those with mutations associated with late-onset disease [43,47]. For this study,

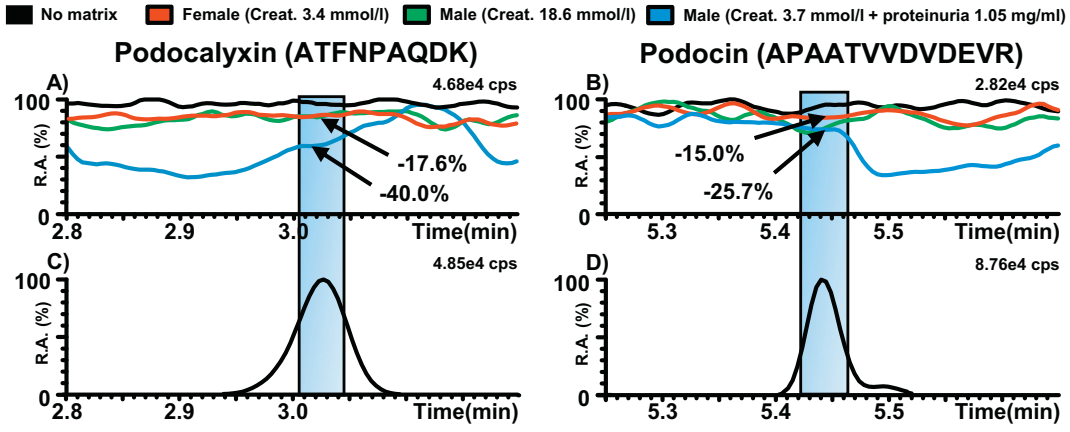

Fig. 2. Matrix effect evaluation for ATFNPAQDK and APAATVVDVDEVR for podocalyxin and podocin peptides, respectively, with 2 urine samples from healthy controls with different creatinine concentrations (3.4 and 18.6 mmol/l) and 1 urine sample from a patient with proteinuria (1.05 mg/ml). A) Ion signal of infused SIL of ATFNPAQDK*; B) Ion infusion of SIL of APAATVVDVDEVR*; C) Ion chromatogram of ATFNPAQDK; D) Ion chromatogram of APAATVVDVDEVR; Infusion concentration = 10 nmol/l; Injection volume = 7.5 µl; Peptide concentration = 5 nmol/l; Infusion flow rate = 20 µl/min; R.A.: Relative abundance (%).

Table 2
Demographic and clinical characteristics of pathological controls, women with preeclampsia and Fabry patients. Male and female controls are also included. Median values are shown.

	Age	eGFR	DBP	SBP	GA	DA	α-galA activity
	(years)	(ml/min/1.73m^2)	(mmHg)	(mmHg)	(weeks)	(weeks)	(nmol/h/mg prot.)
Fabry groups							
CM (n = 10)	41 (25–63)	n/m	n/m	n/m	n/a	n/a	n/m
CF (n = 10)	39 (22–67)	n/m	n/m	n/m	n/a	n/a	n/m
TFM (n = 11)	38 (23–62)	82 (29–121)	79 (60–89)	117 (100–149)	n/a	n/a	4.0 (0.9–6.3)
TFF (n = 6)	59 (49–70)	81 (53–92)	77 (68–90)	112 (100–142)	n/a	n/a	33.0 (11.0–68.0)
UFM (n = 2)	39 (33–44)	77 (69–86)	n/m	n/m	n/a	n/a	3.2 (2.4–4.0)
UFF (n = 10)	48 (19–77)	88 (55–138)	84 (54–88)	132 (96–164)	n/a	n/a	23.3 (0.0–61.0)
Preeclampsia groups							
CF (n = 10)	39 (22–67)	n/m	n/m	n/m	n/a	n/a	n/m
NP (*n* = 15)	31 (20–37)	120 (78–120)	74 (57–84)	120 (97–132)	31.2 (28.0–36.6)	41.9 (38.0–47.6)	n/a
GD (n = 9)	30 (28–40)	120 (107–120)	68 (50–89)	103 (90–132)	39.1 (32.0–45.6)	41.4 (39.0–45.1)	n/a
HP (n = 8)	27 (22–35)	120 (94–120)	87 (84–98)	131 (116–150)	39.4 (34.0–43.6)	38.4 (37.0–43.3)	n/a
PE (*n* = 13)	27 (21–37)	116 (86–120)	90 (82–118)	148 (135–180)	38.1 (31.9–41.7)	37.7 (31.4–44.6)	n/a

eGFR: estimated glomerular filtration rate; DBP: diastolic blood pressure; SBP: systolic blood pressure; GA: gestational age; DA: delivery age; CM: control males; CF: control females; TFM: ERT-treated Fabry males; TFF: ERT-treated Fabry females; UF: Untreated Fabry males; UFF: Untreated Fabry females; NP: women with normotensive pregnancies; GD: women with gestational diabetes; HP: women with hypertensive pregnancies; PE: women with preeclampsia; n/m: not measured; n/a: not applicable; (): minimum and maximum value in a group.

Fabry subjects with classical mutations were observed in our Fabry patient groups (Supplementary material Table S-5).

Areas under the ROC curves (AUCs) were used to assess the efficacy of podocalyxin and podocin levels to discriminate patients from controls. Our results show that podocalyxin concentrations were 16.3 (from 7.9 to 53.7) times higher than podocin concentrations. For the Fabry groups (UFM, UFF, TFM, TFF), results showed that the podocalyxin concentrations were significantly higher in control females (CF) than males (CM) ($p = .041$). A significantly higher concentration of podocin for Fabry males was observed compared to CM. In fact, the AUC was slightly higher in UFM ($p = .039$; AUC = 0.95) than TFM ($p = .019$; AUC = 0.79), but no significant differences were observed between treated and untreated Fabry males ($p > .05$). Regarding Fabry females, podocalyxin and podocin levels were only significantly elevated in untreated females with an AUC for podocalyxin slightly higher ($p = .016$; AUC = 0.82) than podocin ($p = .048$; AUC = 0.75). The increased of podocyte proteins in urine may be consistent with the accumulation of Gb_3 in podocytes of Fabry patients [10].

For the preeclampsia groups (NP, GD, HP, PE), podocalyxin and podocin levels were significantly higher in pregnant women compared to non-pregnant women (Podocalyxin: $p < .001$; Podocin: $p = .016$). We thus selected the normotensive pregnant (NP) women group as normal reference controls for all pregnant women. A significant elevation of podocalyxin levels was observed for hypertensive (HP) and women with preeclampsia (PE) compared to the normal reference group (NP). We observed that AUC values were slightly higher for the PE group ($p < .001$; AUC = 0.95) compared to the HP group ($p = .005$; AUC = 0.87). Higher concentrations of podocin were also observed, but only for PE ($p < .001$; AUC = 0.96). Biomarker levels in the PE group were significantly higher than HP for both podocalyxin and podocin. No significant differences were observed between NP and GD ($p > .05$). Our results for preeclampsia women are consistent with recent findings showing an elevation of urinary extracellular vesicles and in solution podocyte protein in women with preeclampsia [48].

3.4. Normal values for podocalyxin and podocin in random urine samples

Normal values were defined as the 95th percentile measured in control groups to assess the sensitivity and specificity of the podocalyxin and podocin values obtained for each group (Table 4). For the

Table 3
Total Gb_3, lyso-Gb_3, podocalyxin, podocin, proteinuria and albuminuria median values measured in urine and normalized to creatinine according to each study group.

	Podocalyxin/Creat.	Podocin/Creat.	Prot./Creat.	Alb./Creat.	Total Gb_3/Creat.	Lyso-Gb_3/Creat.
	(pmol/mmol)	(pmol/mmol)	(mg/mmol)	(mg/mmol)	(μg/mmol)	(pmol/mmol)
Fabry groups						
CM (n = 10)	25.3 (16.0–39.3)	0.0 (nd-3.0)	10.0 (5.1–29.4)	0.5 (0.2–1.5)	5.5 (2.2–39.7)	nd
CF (n = 10)	32.6 (18.7–46.1)	0.0 (nd-3.9)	15.1 (4.0–47.6)	0.6 (0.3–2.4)	7.0 (3.5–30.6)	nd
TFM (n = 11)	34.8 (21.2–72.0)	3.8 (nd-7.4)	73.5 (4.5–283.8)	47.7 (0.3–271.4)	30.4 (6.4–789.0)	52.2 (3.4–683.7)
TFF (n = 6)	42.9 (22.8–59.3)	0.8 (nd-6.7)	35.5 (6.7–76.5)	15.4 (0.3–55.3)	23.4 (7.2–70.7)	16.3 (7.0–38.1)
UFM (n = 2)	36.8 (28.0–45.5)	4.7 (3.0–6.4)	130.0 (122.0–138.0)	116.1 (109.0–123.2)	626.8 (560.6–692.9)	546.2 (384.8–707.5)
UFF (n = 10)	54.5 (28.4–113.5)	3.2 (nd-8.1)	18.6 (6.3–55.6)	1.8 (1.0–8.4)	77.0 (13.8–253.3)	19.5 (nd-143.7)
Preeclampsia groups						
CF (n = 10)	32.6 (18.7–46.1)	0.0 (nd-3.9)	15.1 (4.0–47.6)	0.6 (0.3–2.4)	7.0 (3.5–30.6)	nd
NP (n = 15)	56.4 (36.4–79.0)	3.6 (nd-6.5)	12.4 (6.2–29.4)	0.5 (0.3–11.0)	n/m	n/m
GD (n = 9)	57.4 (39.9–116.6)	5.1(1.3–8.3)	11.7 (6.3–32.3)	0.6 (0.3–1.3)	n/m	n/m
HP (n = 8)	85.0 (55.6–205.8)	4.4 (nd-14.6)	11.5 (8.4–72.6)	1.2 (0.4–39.8)	n/m	n/m
PE (n = 13)	232.6 (55.0–792.4)	8.4 (4.4–23.8)	67.9 (11.6–1544.4)	40.8 (1.8–888.9)	n/m	n/m

Prot.: proteinuria; Alb.: albuminuria; Creat.: creatinine; CM: control males; CF: control females; TFM: ERT-treated Fabry males; TFF: ERT-treated Fabry females; UFM: untreated Fabry males; UFF: untreated Fabry females; NP: women with normotensive pregnancies; GD: women with gestational diabetes; HP: women with hypertensive pregnancies; PE: women with preeclampsia; n/m: not measured; nd: not detected.

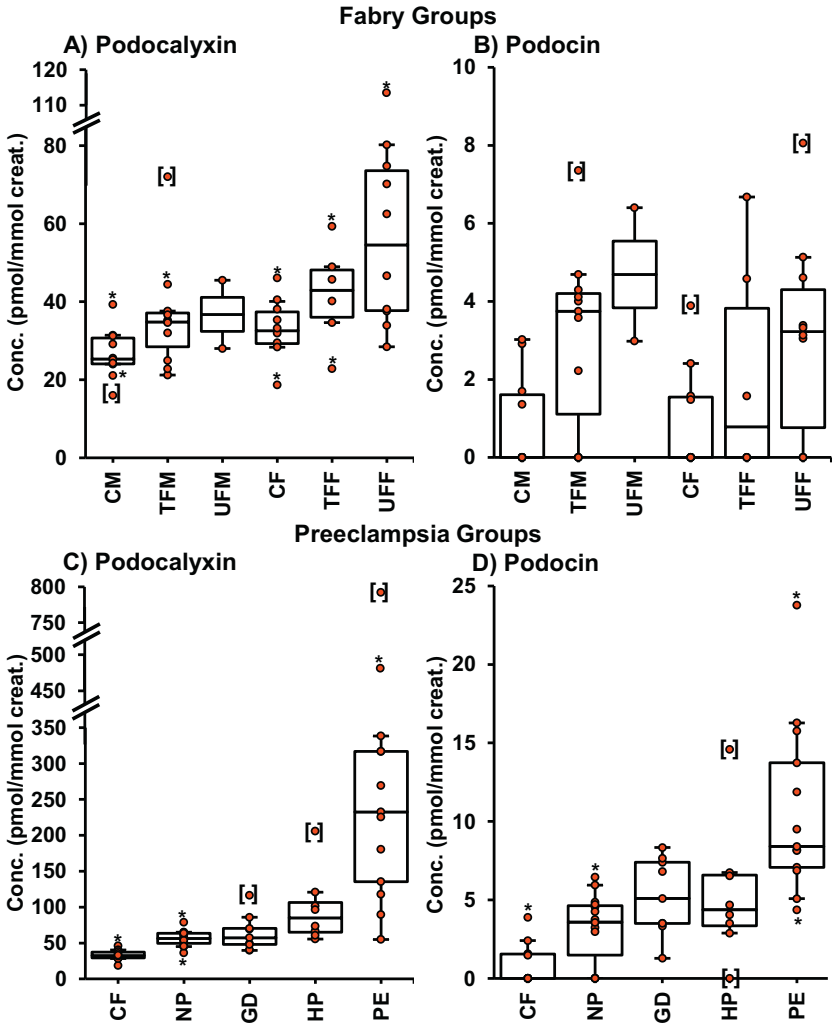

Fig. 3. Box plots of the urinary levels measured of podocalyxin (ATFNPAQDK) and podocin (APAATVVDVDEVR) normalized to creatinine (pmol/mmol creatinine) in Fabry and preeclampsia groups by using cleavable peptide standards. Upper box plots represent normalized urinary levels measured of: A) Podocalyxin and B) Podocin for control males (CM), ERT-treated Fabry males (TFM), untreated Fabry males (UFM), control females (CF), ERT-treated Fabry females (TFF) and untreated Fabry females (UFF) as Fabry groups. Lower box plots represent normalized urinary levels of: C) Podocalyxin and D) Podocin for control females (CF), women with normotensive pregnancies (NP), women with gestational diabetes (GD), women with hypertensive pregnancies (HP); and women with preeclampsia (PE) in the preeclampsia groups. The lower and upper limits shown by the box plots are the 25th and 75th percentiles. Center horizontal box line is the median. The whiskers correspond to the highest and lowest non outlier values. Values over 1.5 times the range between the median and the third quartile over the upper box limit and over 1.5 times the range between the median and the first quartile under the lower box limit represent outliers (*). Values over 3 times the range between the median and the third quartile over the upper box limit and over 3 times the range between the median and the first quartile under the box limit represent extreme outliers [ ].

Fabry groups, reference values were established from healthy controls according to gender, and the normotensive pregnancy group (NP) was the reference control group for the preeclampsia groups due to the higher level of podocalyxin and podocin compared to the non-pregnant control group. The specificity of podocalyxin and podocin was 93.3% for the preeclampsia groups compared to the NP reference group, and 90% for the Fabry groups compared to controls. The sensitivity of podocalyxin was equal or greater than the sensitivity of podocin in DG, HP, PE, TFF and UFF. Interestingly, the sensitivity of podocin was higher than the sensitivity of podocalyxin in TFM and UFM. However, the sensitivity of both proteins were greater in PE and untreated Fabry patients compared to healthy controls and ERT-treated Fabry patients. We observed that the sensitivities of the measurements of podocalyxin and podocin in some groups of subjects were low probably due to the reduced number of patients involved in these groups.

3.5. Correlations with symptoms

Correlations were found between podocalyxin and podocin levels and different clinical parameters for the Fabry groups (CF, CM, TFM, UFM, TFF, UFF) and the preeclampsia groups (NP, GD, HP, PE) (Table 5) using the non-parametric Spearman test (significant correlation: $p < .05$). In the preeclampsia groups, blood pressure, albuminuria and proteinuria levels positively correlated with podocalyxin and podocin levels, while gestational delivery age negatively correlated. In the Fabry groups, urinary lyso-Gb_3 levels showed positive correlations with podocalyxin and podocin levels, while total urinary Gb_3 levels

Table 4
Normal values, sensitivity and specificity for podocalyxin and podocin. Normal values correspond to the 95th percentile from NP (n = 15) for pregnant women samples and healthy controls CM (n = 10) and CF (n = 10) for the Fabry groups.

	Podocalyxin	Podocin
Fabry groups		
Normal values (unit)	♂: 35.7; ♀: 43.6 (pmol/mmol creat.)	♂: 3.0; ♀: 3.2 (pmol/mmol creat.)
Sensitivity		
TFM (n = 11)	36.4%	63.6%
TFF (n = 6)	50.0%	33.3%
UFM (n = 2)	50.0%	100.0%
UFF (n = 10)	60.0%	50.0%
Specificity	90.0%	90.0%
Preeclampsia groups		
Normal values (unit)	♀ pregnant: 69.4 (pmol/mmol creat.)	♀ pregnant: 6.1 (pmol/mmol creat.)
Sensitivity		
DG (n = 9)	44.4%	44.4%
HP (n = 8)	62.5%	37.5%
PE (n = 13)	92.3%	84.6%
Specificity	93.3%	93.3%

CM: control males; CF: control females; TFM: ERT-treated Fabry males; TFF: ERT-treated Fabry females; UFM: untreated Fabry males; UFF: untreated Fabry females; NP: women with normotensive pregnancies; GD: women with gestational diabetes; HP: women with hypertensive pregnancies; PE: women with preeclampsia; ♀: female; ♂: male.

Table 5
Correlations established for podocalyxin and podocin levels and clinical parameters observed in the Fabry and preeclampsia groups using the non-parametric Spearman test (Fabry groups: CF, CM, TFM, UFM, TFF, UFF; Preeclampsia groups: CF, NP, GD, NP, PE). Values: Spearman rho.

	Podocalyxin	Podocin
Fabry groups		
Podocalyxin (pmol/mmol creat.) (n = 49)	n/a	0.395**
Podocin (pmol/mmol creat.) (n = 49)	0.395**	n/a
Urinary lyso-Gb_3 (pmol/mmol creat.) (n = 49)	0.306*	0.522**
Total urinary Gb_3 (μg/mmol creat.) (n = 49)	0.298*	n/s
Preeclampsia groups		
Podocalyxin (pmol/mmol creat.) (n = 45)	n/a	0.804**
Podocin (pmol/mmol creat.) (n = 45)	0.804**	n/a
Proteinuria (mg/mmol creat.) (n = 45)	0.607**	0.509**
Albuminuria (mg/mmol creat.) (n = 45)	0.729**	0.636**
DA (weeks) (n = 45)	−0.524**	−0.439**
DBP (mmHg) (n = 45)	0.701**	0.556**
SBP (mmHg) (n = 45)	0.729**	0.538**

DA: delivery age; DBP: diastolic blood pressure; SBP: systolic blood pressure; n/s: not significative; n/a: not applicable.
* $p < .05$.
** $p < .01$.

showed a positive correlation only with podocalyxin levels. The rho correlation Spearman values were higher for the preeclampsia groups compared to the Fabry groups.

4. Conclusions

To our knowledge, this is the first time that a validated UPLC-MS/MS method for simultaneous quantitation of podocalyxin and podocin levels in urine using cleavable peptide standards is reported. Liquid chromatography parameters were optimized and specific selected peptides were used to independently assess both biomarkers and to evaluate podocyturia in patients with potential renal damage. Only 1 ml of supernatant of random urine samples was necessary to establish that podocalyxin levels were significantly higher than podocin levels in patients under study and particularly higher in urine samples from females compared to males. Podocalyxin and podocin levels in women with preeclampsia were significantly higher than controls. Podocalyxin levels were more sensitive for women with preeclampsia and untreated Fabry females compared to podocin levels. By contrast, podocin appeared to be a more appropriate biomarker for Fabry males not on ERT compared to podocalyxin based on the sensitivity and the specificity of the results; however, this might also be due to the low concentrations of biomarkers in this latter group. Podocalyxin and podocin levels correlated well with several clinical parameters for women with preeclampsia and Fabry patients evaluated in this study. Interesting correlations were observed between lyso-Gb_3, Gb_3 and podocyte proteins, but the limited number of recruited patients may affect the results and is considered as a limitation for this study. According to our results, this mass spectrometry methodology is useful as a clinical investigative tool to quantify podocyturia proteins simultaneously in the urine of targeted patients. It also offers an alternative tool to evaluate kidney involvement earlier for affected patients.

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Appendix A. Supplementary data

The Supplementary material is available free of charge on the *Clinical Chimica Acta* Publications website.

Page S-1 shows Supplementary protocols for recombinant proteins and HSA peptide analysis, as well as Supplementary Tables and Figures. Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.03.1615.

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