Metabolic profiling of human plasma and urine in chronic kidney disease by hydrophilic interaction liquid chromatography coupled with time-of-flight mass spectrometry: a pilot study

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Abstract

Chronic kidney disease (CKD) is often characterized by a progressive loss in renal function over a period of months or years and by the accumulation of uremic retention solutes in the body. Current biomarkers, such as serum creatinine or urinary albumin, lack the sensitivity for early detection of CKD, which is primordial towards disease management. In the search for new uremic retention solutes and/or biomarkers of CKD, a hydrophilic interaction liquid chromatography time-of-flight mass spectrometric (HILIC-TOF MS) platform was developed. Urine and plasma samples from CKD patients at stage 3 (n=20), at stage 5 not yet receiving dialysis (n=20) and from healthy controls (n=20) were monitored in both positive and negative electrospray ionization mode. The validity of the metabolomics dataset was ensured by quality control (QC) samples. Data were treated with XCMS followed by multivariate statistical analysis. Differentiation was achieved between the metabolic profile of the CKD patients and healthy controls. Moreover, 4 metabolites that showed a significant increase or decrease throughout the different stages of CKD, i.e. cinnamoylglycine, glycoursodeoxycholic acid, 2-hydroxyethane sulfonate, and pregnenolone sulphate, could be identified by the use of authentic standards, the latter three of which are newly detected uremic retention solutes.

Keywords Chronic kidney disease • hydrophilic interaction liquid chromatography • metabolomics • uremic retention solutes • urine and plasma

1. Introduction

Chronic kidney disease (CKD) is a worldwide public health problem often characterized by a gradual loss of kidney function over time [1]. A multitude of compounds which are normally secreted by the healthy kidneys into the urine, are no more or insufficiently removed and accumulate in the body. These substances are called uremic retention solutes. If these compounds exert biological or biochemical activities, they are referred to as uremic toxins [2-4]. In 2003, the European Uremic Toxin (EUTox) Work Group composed an encyclopedic list of 90 uremic retention solutes, divided into 3 classes based on their physico-chemical properties [5]. In 2012, an update of the list provided 56 newly identified solutes. Nevertheless, many uremic retention solutes remain unknown. CKD is classified into 5 stages, with stage 1 being the mildest and stage 5 (end stage) being a severe illness with poor life expectancy if untreated [6]. Monitoring CKD activity requires non-invasive, specific and sensitive biomarkers that provide clinicians with information correlating with pathophysiologic processes occurring within the kidney. Current biomarkers of CKD and its progression that are in widespread clinical use, such as serum creatinine and albuminuria, have limitations in serving these goals and reliance on these biomarkers may result in an extensive time lapse between the start of the disease and the moment when alarm signs are prominent enough to incite therapeutic interventions [7-12]. Hence, the search for new relevant biomarkers should be continued to better stratify patients with CKD.

Metabolomics, a recent systems biology approach, complements the genomic, transcriptomic, and proteomic efforts to characterize a biological system and can be regarded as the end point of the “-omics”-cascade. Since metabolites represent end products, metabolomics holds the promise of providing an integrated physiologic phenotype of a system [13,14]. The main analytical techniques in metabolomics studies involve mass spectrometry (MS), usually preceded by a chromatographic separation step, and nuclear magnetic resonance spectroscopy

(NMR). In the search for novel biomarkers of CKD, NMR [15-17] and hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS) [18-20], liquid chromatography-mass spectrometry (LC-MS) [21-23] and capillary electrophoresis-mass spectrometry (CE-MS) [24-27] have been applied. As no single analytical technique is entirely competent in covering the broad metabolic picture, combining multiparallel technologies, has become indispensable, aiming at a comprehensive metabolome coverage. Recently, several studies on CKD have combined LC-MS and GC-MS to enlarge this coverage [28,29]. Within LC-MS-based metabolomics studies the metabolome coverage can be extended by combining multiple LC separation modes. The majority of the LC-MS-based studies employ reversed phase liquid chromatography (RPLC). The use of reversed phase columns provides efficient separation and retention of relatively nonpolar metabolites across a large molecular weight range. Polar metabolites, being mainly primary metabolites, elute in the column void or early in the chromatographic run. The latter species represent metabolite classes of high significance, such as amino acids and organic acids, which are directly involved in the normal growth, development, or reproduction of an organism and are thus important for the diagnosis of diseases. Hydrophilic interaction chromatography (HILIC) has become increasingly popular for the analysis of polar metabolites and several metabolomics studies have confirmed that the addition of HILIC is a useful tool to increase the metabolome coverage [30-34]. However, no HILIC-based metabolomics studies have been reported in the search for novel uremic retention solutes or potential biomarkers of CKD. Therefore, the goal of this study is to develop and apply a HILIC-time-of-flight (TOF) MS metabolic platform in the search for novel uremic retention solutes/potential biomarkers by comparing urine and plasma from a healthy control group and two patient groups suffering from different stages of CKD, i.e. CKD stage 3 (CKD3) and CKD stage 5 (CKD5).

2. Materials and methods

2.1. Chemicals

Water (LC-MS grade), methanol (LC-MS grade), ammonium acetate (ULC-MS grade) and formic acid (ULC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Metabolite standards were obtained from The Metabolomics Innovation Centre (TMIC, Edmonton, Canada).

2.2. Study samples

Urine samples were obtained by centrifugation of fresh urine from 40 patients diagnozed with CKD and 20 healthy controls at 1800 rpm for 10 min at room temperature. Aliquots of 1000 µl were stored at -80°C until analysis. Blood was sampled by venipuncture from the same study group using K2EDTA Vacutainer™ tubes (Becton Dickinson, San Jose, CA, USA) after informed consent. Plasma was obtained by centrifugation, immediately after sampling, at 3,000 rpm for 10 min at room temperature. Aliquots of 500 µl plasma were stored at -80°C until analysis. The clinical characteristics of the included patients are tabulated in Table 1. From the 40 patients included, 20 were classified in CKD stage 3 (CKD3) (estimated glomerular filtration rate - eGFR - 30-60 mL/min/1.73 m2 body surface) and 20 in CKD stage 5 not receiving dialysis (CKD5) (eGFR < 15 mL/min/1.73 m2), based on their glomerular filtration rate estimated from the serum creatinine values as prescribed by the KDOQI guidelines. The underlying etiology of CKD was of vascular (n=8), glomerular (n=5) or interstitial (n=5) origin, polycystic kidney disease (n=5), nephrectomy (n=8), transplant failure (n=4), other (n=4) or unknown (n=1).

2.3. Sample preparation

2.3.1. Sample preparation: urine

The frozen urine samples were thawed, and were prepared by adding 180 µL of water to 60 µL of urine in Eppendorf tubes, briefly vortexing, and centrifuging at 13,000 rpm for 10 min. Subsequently, 180 µL was dried under nitrogen at room temperature and re-dissolved in 120 µL of the initial mobile phase (see 2.4).

2.3.2. Sample preparation: plasma

The frozen plasma samples were thawed. Subsequently, 150 µL of plasma was transferred to Eppendorf tubes and 300 µL of ice-cold methanol (-20°C) was added. After vortex mixing during 10 seconds, samples were placed at -20°C for 20 minutes. Afterwards, the samples were centrifuged for 10 minutes at 13,000 rpm and 300 µL of the supernatant was dried under nitrogen at room temperature. The dried extracts were re-dissolved in 150 µL of the initial mobile phase (see 2.4) and transferred to a new Eppendorf tube. The samples were centrifuged for 10 min at 13,000 rpm, after which 150 µL of supernatant was transferred to a vial with glass insert for LC-MS analysis.

2.3.3. Preparation of QC samples

A quality control (QC) pool was constructed by collecting 100 µL of all the study samples. Subsequently, this QC pool was divided into aliquots to acquire representative QC samples. QC samples were prepared simultaneously along with study samples and were analyzed throughout the LC-MS analysis every 5 study samples. Since these samples do not contain any biological variability, they can be considered as technical replicates. For both plasma and urine, study and QC samples were prepared in random order.

2.4. Liquid chromatography-mass spectrometry conditions

All samples were analyzed on a 1290 Infinity LC coupled to a 6230 TOF MS (Agilent Technologies), equipped with a Jetstream electrospray ionization (ESI) source as interface. Separation was performed on an Acquity UPLC BEH HILIC column (1.7 µm, 2.1 mm x 150 mm) with an Acquity UPLC BEH HILIC VanGuard precolumn (1.7 µm, 2.1 mm x 5 mm). The mobile phase consisted of (A) 0.1% formic acid in 50 mM ammonium acetate and (B) 0.1% formic acid in acetonitrile. Elution was carried out with a gradient starting from 2% A to 20% A in 30 min, followed by an increase to 100% A and re-equilibration. The flow rate was 0.3 mL/min and the injection volume 10 µL. The column temperature and the autosampler temperature were kept at

40°C and 4° C, respectively. The MS instrument was operated in both positive and negative ESI modes. Needle voltage was optimized to 4 kV in positive ESI mode and -3.5 kV in negative ESI mode. The drying and sheath gas temperatures were set to 325°C and the drying and sheath gas flow rates were set to 8 and 7 L/min, respectively. Data were collected in centroid mode from m/z 50–1,200 at an acquisition rate of 1 spectrum/sec in the extended dynamic range mode (2 GHz). To maintain mass accuracy during the analysis sequence, a reference mass solution was used containing reference ions (121.0508 and 922.0097 for positive ESI mode, and 112.9856 and 1033.9881 for negative ESI mode). The TOF instrument was tuned using the ESI-L low concentration tuning mix (Agilent Technologies) prior to the analysis sequence. The LC-MS analysis was performed in one batch, separately for positive ESI and negative ESI measurement. Each analysis sequence started with 2 blank runs and 6 conditioning samples (QC samples). All study samples were analyzed in randomized order in both ionization modes, with QC samples (n=12) analyzed every 5 study samples. All instruments were controlled by MassHunter Workstation Acquisition 4.0 (Agilent Technologies). Fig. 1 displays representative chromatograms (positive ESI mode) of plasma obtained from (a) a healthy control and (b) a CKD5 patient.

2.5. Data Analysis

The LC-MS total ion chromatogram (TIC) data were exported to mzData format data files by MassHunter Qualitative Analysis B.04.00 (Agilent Technologies) and pairs of sample groups were subsequently processed by XCMS software using default parameters [35]. XCMS software (version 1.34.0) running under the R package (version 2.15.3), incorporates nonlinear retention time alignment, matched filtration, automatic peak detection and peak matching and is freely available under an open-source license. Subsequently, all sample groups were analyzed pairwise (healthy versus CKD3, healthy versus CKD5, CKD3 versus CKD5) in R, using univariate statistics based on unpaired Mann-Whitney testing with Benjamini-Hochberg

corrected p-values to control the false discovery rate (FDR). Next, the data were normalized to the total intensity of components that are common to all samples, which is also called MS “total useful signal” (MSTUS) [36]. A second order analysis was performed to identify metabolites that are up- or downregulated across the different CKD stages. Therefore, the processed XCMS output files were used as input for metaXCMS (version 0.1.20), where they were realigned, statistically evaluated and compared for shared differences. MetaXCMS is freely available as an open-source R-package that includes a graphical user interface [37]. Feature lists were filtered by a fold change (FC) $\geqslant$ 1.5 and corrected p-value (q) $\leqslant$ 0.05 (see 3.3). The second-order comparison was applied using a tolerance of 0.01 m/z and 60 s retention time. Results were visualized as a Venn diagram with the number of common features to all sample groups contained within the intersection. Since this study focused on identifying small molecules that showed an evolution related to the disease progression (healthy > CKD3 > CKD5 or healthy < CKD3 < CKD5), only features were retained that were upregulated or downregulated from healthy to CKD5. The number of features was further reduced by removing features that were not present in at least 75 % of one of the sample groups. The XCMS algorithm does not classify spectral ions originating from the same compound. Hence, the resulting feature list encloses multiple ions for each individual metabolite detected. Therefore, the CAMERA package, which is freely available from the Bioconductor repository, was used for grouping related features and for the annotation of ion species [38]. Data were processed with CAMERA functions in the following order xsAnnotate, groupFWHM, groupCorr, findIsotopes, and findAdducts using default parameters. Data were uploaded into freely available MetaboAnalyst software to construct PCA plots. Molecular formulas were generated by Find by Molecular Feature and Generate Formulas in the MassHunter Qualitative Analysis B.04.00 software. The accurate mass and molecular formula were then matched to metabolites via searching of the on-line Metlin database. Finally, identification was confirmed with commercially available authentic standards.

3. Results and discussion

3.1. Quality of analysis

In order to allow retention and satisfactory chromatography of as much polar solutes a possible in HILIC a gradient method was developed allowing both retention of the less polar solutes (at 2% aqueous phase) and also elution of the most polar ones (at 20% aqueous phase) within a reasonable analysis time on the used stationary phase. The separations were performed on a long (15 cm) sub-2 micron type of column to allow reaching high peak capacities for both the urine and plasma samples. The QC data, obtained on the developed methodology, were subsequently examined post-run in both a targeted and a non-targeted way for evidence of changes during sequences. Targeted monitoring was performed by determining the error of the measurement on signal intensity (peak area), retention time and mass accuracy for a list of randomly selected metabolites. Table 2 summarizes the results of this targeted validity verification. Peak area fluctuations, originating from both the sample preparation step and the LC-MS analysis, are typically below 15% relative standard deviation (RSD, n=12) and are generally better for urine than for plasma. Chromatographic retention time reproducibility is in general satisfactory and limited to a few RSD%. Representative total ion chromatograms by HILIC-MS are shown in Fig. 1. High mass accuracy is expected for the TOF instrument (< 5 ppm), which is advantageous for identification purposes since the greater the accuracy the lower the number of molecular formula matches. We only found one out of twelve mass accuracies exceeding 5 ppm for the list of randomly selected metabolites, which indicates overall acceptable accuracies. Apart from this targeted approach, the reproducibility of the applied metabolomics analysis was examined in a more comprehensive way by calculating the error on all detected features in the QC samples and representing the acquired RSD distribution as depicted in Fig. 2. For urine in positive mode 71.12% of all features show RSD values below 15% and 89.29% of all features had an RSD below 30%, which can be defined as the upper limit for untargeted or discovery metabolomics analysis [32]. Urine analysis in negative ESI mode

displayed 70.43% of all features with a RSD value below 15%, while 92.52% of the features had an RSD value below 30%. For plasma in positive ESI mode 36.35% of all features showed RSD values below 15% and 73.91% of all features had an RSD below 30%. Plasma analysis in negative mode displayed 46.62% of all features with RSD values below 15%, while 73.91% of the features had a RSD below 30%. Hence, the reproducibility of the urine analyses is better than the reproducibility of the plasma analyses.

3.2. Revealing differential metabolites

PCA was used as a first exploratory step in the data-processing pipeline. Grouping of the samples originating from the different CKD stages and controls can be seen from the score plots in Fig. 3. Healthy controls are clearly separated from CKD5 samples. Nevertheless, there is overlap between CKD3 samples and the other sample groups, for both urine and plasma.

Data analysis in metabolomics experiments boils down to reducing complex data matrices to a list of biologically relevant metabolites. Table 3 summarizes the feature reduction throughout the successive data-processing steps.

Creatinine, a clinically widespread biomarker of CKD, was well retained in the HILIC mode and was confirmed as an uremic metabolite. The fold changes of creatinine and several well-known uremic toxins are listed in the upper part of Table 4. In general, fold changes were more substantial for protein bound solutes, such as indoxylsulfate or p-cresylsulfate, compared to the current marker serum creatinine, especially during the early stages of CKD (CKD3 vs H). As expected fold changes of creatinine were more prominent in plasma than in urine, where they are a reflection of daily generation. In an attempt to discover early markers for CKD or new uremic retention solutes, restrictions were set on the fold changes based on the relative difference in creatinine between CKD3 patients and healthy controls in plasma. Only features with fold changes ≥ 1.5 that showed a significant increase or decrease throughout the different stages of CKD were withdrawn. Several features were found to be significant with the required

increasing or decreasing trend throughout CKD as demonstrated in Table 3. In urine analyzed in positive ESI, 15 features were going up, while 59 features were decreasing with CKD progression. Negative ESI unraveled 5 features that were upregulated and 31 features that were downregulated with CKD progression in urine. In plasma analyzed in positive ESI, 17 features were increasing, while 1 feature was decreasing with CKD progression. Negative ESI unraveled 40 features that were upregulated in plasma, next to 11 features that were downregulated with CKD progression.

3.3. Uremic retention solutes in CKD

Currently, the process of metabolite identification in non-targeted metabolomic studies is a significant bottleneck in deriving biological knowledge from metabolomic studies. The Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI) [39] has defined four different levels of metabolites identification confidence, with level 1 being the highest confidence level corresponding to confidently identified compounds by comparison of two or more orthogonal properties with an authentic chemical standard analyzed under identical analytical conditions; level 4 on the other hand is the lowest confidence level corresponding to unknown compounds. In the present study, metabolites were identified by commercially available authentic standards, based on retention time and accurate mass, which is in accordance with confidence level 1 defined by the MSI. Confidently identified metabolites are presented in the lower part of Table 4. Glycoursodeoxycholic acid (GUDCA) and 2-hydroxyethane sulfonate, which have not been reported in the context of CKD yet, were downregulated in urine. Glycoursodeoxycholic acid is an acyl glycine and a secondary bile acid (BA)-glycine conjugate. Primary BAs are synthesized and conjugated in hepatocytes, followed by excretion into bile and the intestinal tract. Gut microorganisms generate secondary BAs by deconjugation and dehydroxylation. Upon reuptake by intestinal transporters, BAs are re-conjugated in the liver to complete the enterohepatic cycle. BAs can also be filtered in the kidney through the glomerulus,

followed by urinary excretion. The solute carrier (SLC) family 10 (SLC10A1 and SLC10A2) is involved in the influx transport of bile acids [40]. It was demonstrated that GUDCA suppresses the production of the proinflammatory cytokines TNF-α and interleukin (IL)-1β and prevents nerve cell death induced by unconjugated bilirubin (UCB) [41,42]. Moreover, GUDCA was shown to have antioxidant properties [43]. Unlike the other metabolites, glycoursodeoxycholic acid was not detected in all subjects, but its fold changes were the highest of the identified metabolites. 2-Hydroxyethane sulfonate (isethionic acid) is a short chain alkane sulfonate involved in the taurine and hypotaurine metabolism [44]. Cinnamoylglycine was upregulated in plasma. Cinnamoylglycine is known as an N-acyl glycine metabolite of cinnamic acid [45]. It allows the transport and elimination of phenylpropanoic acids, a pathway probably similar to the elimination of toluene or benzoic acid as hippuric acid. The accumulation of cinnamoylglycine in CKD has recently been reported in literature [23,29,46]. In a study on colon-derived uremic solutes cinnamoylglycine was shown to be less prominent in patients without a colon than in patients with a colon [23]. Finally, pregnenolone sulfate, which elutes quite early in the chromatographic run, was found to decrease in plasma throughout CKD. Pregnenolone sulfate is a steroid sulfate with a plethora of actions and functions [47], but has not yet been linked to CKD in literature. It is not the final product of pregnenolone being sulfated, but it is also the starting point for subsequent steroid synthesis pathways. For this subsequent synthesis it is indispensible that the substrate enters the cytosol to come into contact with the cytosolic localized sulfohydrolases. As it is unlikely that pregnenolone sulfate is capable of easily crossing the plasma membrane with its hydrophilic sulfate moiety, transmembrane transport is facilitated by a variety of transporter proteins, such as the organic anion-transporting polypeptide (OATP-B), nowadays classified as solute carrier organic anion transporter (SLCO2B1) [48,49], and the sodium-dependent organic anion transport (SOAT), a member of solute carrier family 10 (SLC10A6), which belongs to the same family as the influx transporters of bile acids [50-52]. As a neurosteroid, pregnenolone sulfate modulates a variety of ion channels, transporters, and enzymes. The negative

modulation of $GABA_A$ chloride channels [53,54], the positive modulation of glutamate response by NMDA receptors [55] and the activation of TRPM1 as well as TRPM3 channels are well established [56,57]. It is interesting to remark that dehydroisoandrosterone sulfate, which was earlier found to decrease in CKD [28,29], is a substrate of the same transporter proteins as pregnenolone sulfate [52,58] and also acts as an inhibitor of the $GABA_A$ receptor [59] and a positive NMDA modulator [60]. Both NMDA activation and GABAergic inhibition have been linked to uremic encephalopathy [61,62].

This study involves the screening of the metabolites present in urine and in the methanolic supernatant of protein precipitated plasma samples. Note that it cannot be excluded that the latter procedure could in principle still include some losses due to residual protein-binding effects requiring the development of alternative sample manipulation procedures [63].

Conclusion

A quality controlled hydrophilic interaction liquid chromatography time-of-flight mass spectrometric (HILIC-TOF MS) platform was developed and applied to discover uremic retention solutes and/or potential biomarkers of CKD in urine and serum. Several metabolites could be identified that showed a significant increase or decrease throughout the different stages of CKD and fold changes of these especially in early CKD were markedly more prominent than those for serum creatinine, a current marker of kidney failure. One of the known problems with serum creatinine as a renal marker is its moderate changes during the early stages of the disease. This handicap could be overcome by using markers found by this study, alone, or even better in combination. The data currently reported should be validated in larger populations.

This discovery study also shows the potential of HILIC-based metabolomics in the study of CKD. More research is needed to definitely label these solutes as uremic toxins and/or biomarkers of CKD.

To reach both aims described above, targeted analytical methods have to be developed, which allow quantification of the identified metabolites. Such quantitative methods will provide concentration ranges for the targeted compounds rather than fold changes, this will then allow the evaluation of the metabolites' biological activity at relevant concentrations for CKD, and the evaluation of their kinetic behaviour in CKD and during renal replacement therapy. Furthermore, quantitative data at the different and preferably earlier stages of CKD will enable the evaluation of the metabolites' predictive value for the presence of CKD and its relative sensitivity and specificity compared to current markers like creatinine.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. The study protocol was approved by the Ethics Committee of Gent University Hospital, and informed consent was obtained prior to collection of the samples.

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Table 1

Clinical characteristics of the included patients

	Healthy	CKD3	CKD5
Number	20	20	20
Age	33.8 ± 13.6	61.0 ± 14.4	64.3 ± 20.0
Male/Female	9/11	12/8	10/10
BMI[a] (kg/m²)	22.7 ± 3.9	26.9 ± 4.0	26.2 ± 4.4
Syst BP[b] (mm Hg)	125 ± 16	134 ± 24	141 ± 24
Diast BP (mm Hg)	77 ± 10	82 ± 11	83 ± 11
Pulse	69 ± 10	68 ± 8	75 ± 15
CTN[c] (mg/dL)	0.92 ± 0.19	1.49 ± 0.30	4.28 ± 0.93
CRP[d] (mg/L)	0.16 ± 0.17	0.20 ± 0.16	1.44 ± 2.68

[a]BMI: body mass index; [b]BP blood pressure; [c]CTN: creatinine; [d]CRP: C-reactive protein

Table 2

Relative standard deviation (RSD) of area under the curve (AUC) and retention time (RT), and average mass accuracy of randomly selected metabolites measured in QC samples (n=12)

Analysis	Metabolite	Theoretical Mass	Average mass accuracy (ppm)	RT (min)	RSD tR (%)	RSD AUC (%)
1a	Adenosine	180.0634	0.89	7.65	0.17	5.43
1a	Hydroxyindole	267.0968	4.70	7.57	0.56	6.34
1a	Pseudo-uridine	133.0528	1.30	1.03	0.32	2.51
2a	Urea	60.0324	5.47	3.62	0.80	6.18
2a	Creatinine	113.0589	4.77	12.22	0.96	6.88
2a	Panthothenic acid	219.1107	4.50	5.15	1.06	11.74
1b	2-Furoylglycine	169.0375	0.79	3.73	0.28	5.71
1b	4-Hydroxyhippuric acid	195.0532	4.94	3.94	0.46	9.7
1b	Phenylacetylglutamine	264.1110	4.68	15.03	0.36	5.66
2b	Pseudo-uridine	244.0695	3.22	2.49	0.31	11.33
2b	Xanthosine	284.0757	3.17	2.82	0.30	13.81
2b	Hypoxanthine	136.0385	2.68	6.03	0.58	10.13

(1a) urine positive ESI; (1b) urine negative ESI; (2a) plasma positive ESI; (2b) plasma negative ESI

Table 3

Feature reduction throughout data-processing

Data processing step	Feature number			
	urine positive ESI	urine negative ESI	plasma positive ESI	plasma negative ESI
XCMS feature detection (CKD3vsH, CKD5vsH and CKD5vsCKD3)	4,442	3,628	3,481	3,508
Differential features MetaXCMS Filter by corrected p-value ($q \leq 0.05$) and fold change ($FC \geq 1.5$)	1,382	1,126	714	992
MetaXCMS common features (m/z tolerance 0.01 and retention time tolerance 60 s)	94	49	20	76
(H < CKD3 < CKD5 or H > CKD3 > CKD5) and 75% frequency criterion	92	47	20	69
CAMERA	74	36	18	51

Table 4 List of most relevant up or down regulated metabolites identified in this study (represented in order of retention time). The metabolites highlighted in bold are confidently identified metabolites that showed a significant increase or decrease throughout CKD. The other metabolites are creatinine and other well-known uremic retention solutes which were also confirmed in this non targeted study as being highly significant solutes.

Sample type	Metabolite	Formula	Mass[a] (Da)	RT (min)	Freq H	Freq CKD3	Freq CKD5	q (CKD3 vs H)	FC (CKD3 vs H)	q (CKD5 vs CKD3)	FC (CKD5 vs CKD3)	q (CKD5 vs H)	FC (CKD5 vs H)	ESI voltage mode
Urine	***2-Hydroxyethane sulfonate***	*$C_2H_6O_4S$*	*125.999*	*3.92*	*20*	*20*	*20*	*4.51E-02*	*-1.51*	*6.85E-03*	*-2.34*	*2.15E-04*	*-3.49*	-
	Glycoursodeoxycholic acid	*$C_{26}H_{43}NO_5$*	*449.313*	*10.31*	*20*	*15*	*2*	*1.36E-03*	*-3.35*	*1.39E-03*	*-8.20*	*3.52E-05*	*-26.93*	-
	Creatinine	$C_4H_7N_3O$	113.058	12.11	20	20	20	1.38E+00	1.04	3.24E-01	1.06	3.04E-02	1.10	+
Plasma	p-Cresylsulfate	$C_7H_8O_4S$	188.015	0.97	20	20	20	1.15E-02	1.58	5.13E-03	1.62	6.71E-06	2.56	-
	Indoxylsulfate	$C_8H_7NO_4S$	213.010	1.01	20	20	20	4.55E-04	1.72	2.25E-02	1.45	2.13E-05	2.49	-
	Pregnenolone sulfate	*$C_{21}H_{32}O_5S$*	*396.197*	*1.27*	*20*	*20*	*19*	*7.36E-03*	*-1.80*	*1.90E-02*	*-1.52*	*4.53E-04*	*-2.70*	-
	CMPF	$C_{12}H_{16}O_5$	240.100	1.67	20	20	20	2.36E+00	1.14	2.81E-02	1.76	2.30E-02	2.02	-
	Hippuric acid	$C_9H_9NO_3$	179.060	2.41	20	20	20	1.72E-01	1.39	9.19E-03	2.22	1.67E-03	3.08	-
	Cinnamoylglycine	$C_{11}H_{11}NO_3$	205.074	4.05	20	20	20	1.27E-02	1.50	3.15E-05	1.90	8.54E-08	2.86	-
	Creatinine	$C_4H_7N_3O$	113.058	12.22	20	20	20	2.15E-03	1.21	4.88E-13	1.59	2.22E-16	1.92	+

Italics represent downregulated metabolites. [a]Measured mass; RT retention time; Freq frequency; FC fold change; CMPF 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; q FDR corrected p value

Figure Captions

Fig. 1 Representative chromatograms of plasma from (a) healthy control and (b) CKD5 patient. Peak annotation 1: p-cresylsulphate, 2: Indoxylsulphate, 3: CMPF, 4: Hippuric acid, 5: Creatinine, 6: Cinnamoylglycine, 7: *Pregnenolone sulfate,* *: other up- or down-regulated solutes between both plasma sample types.

Fig. 2 Relative standard deviation (RSD) distribution plot displaying the technical repeatability of the LC-MS analysis of urine and plasma in both positive and negative ESI mode. The stability of the feature signals is expressed as RSD values, calculated for each feature as the standard deviation of the peak area in all QC samples divided by the average of the peak area in all QC samples

Fig. 3 Principal component analysis score plots obtained from the urine and serum data. Samples are colour-coded according to their group: healthy (blue); CKD3 (red); CKD5 (green)

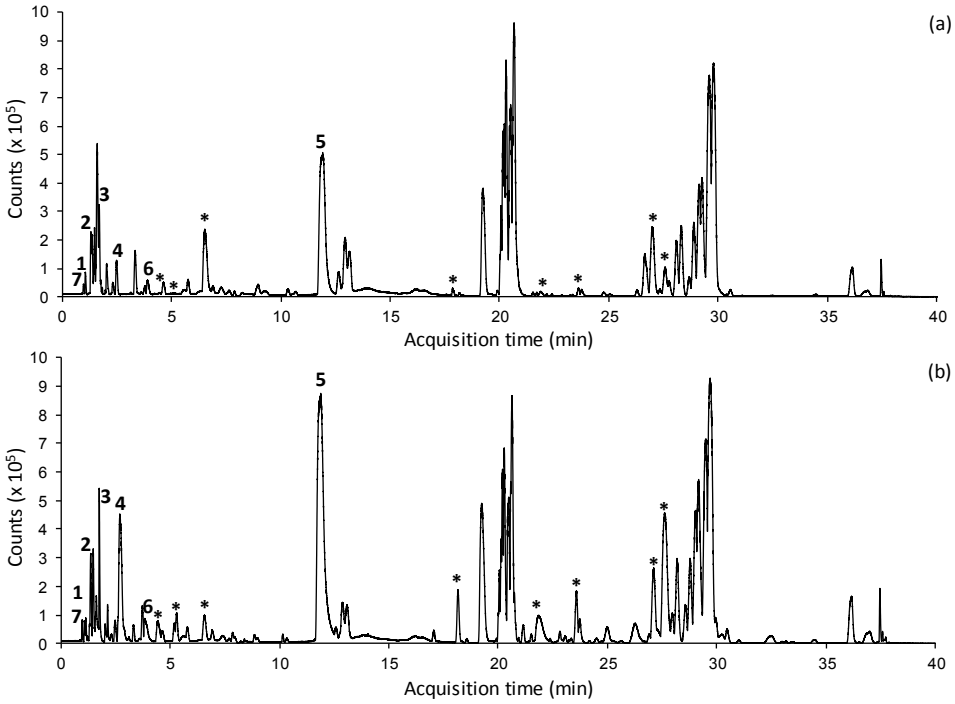
(a)
Counts (x 10^5)
Acquisition time (min)
(b)
Counts (x 10^5)
Acquisition time (min)

Fig. 1

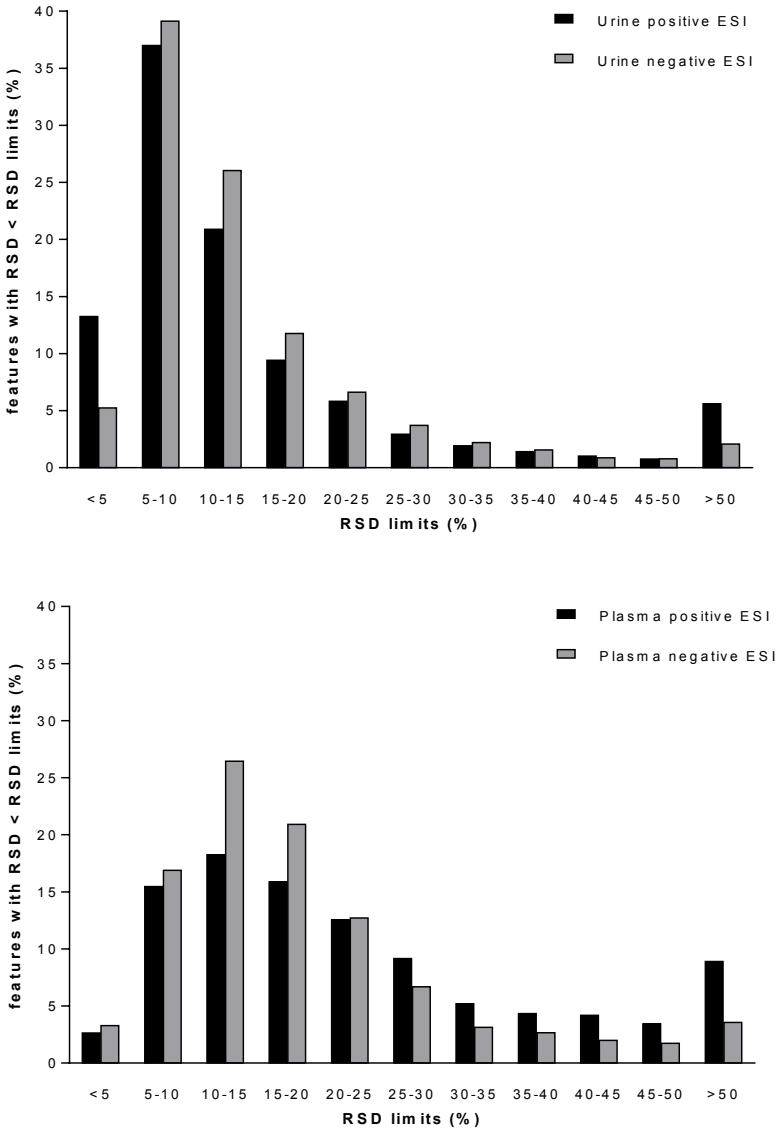
Urine positive ESI
Urine negative ESI
features with RSD < RSD limits (%)
0
5
10
15
20
25
30
35
40
<5
5-10
10-15
15-20
20-25
25-30
30-35
35-40
40-45
45-50
>50
RSD limits (%)
Plasma positive ESI
Plasma negative ESI
features with RSD < RSD limits (%)
0
5
10
15
20
25
30
35
40
<5
5-10
10-15
15-20
20-25
25-30
30-35
35-40
40-45
45-50
>50
RSD limits (%)

Fig. 2

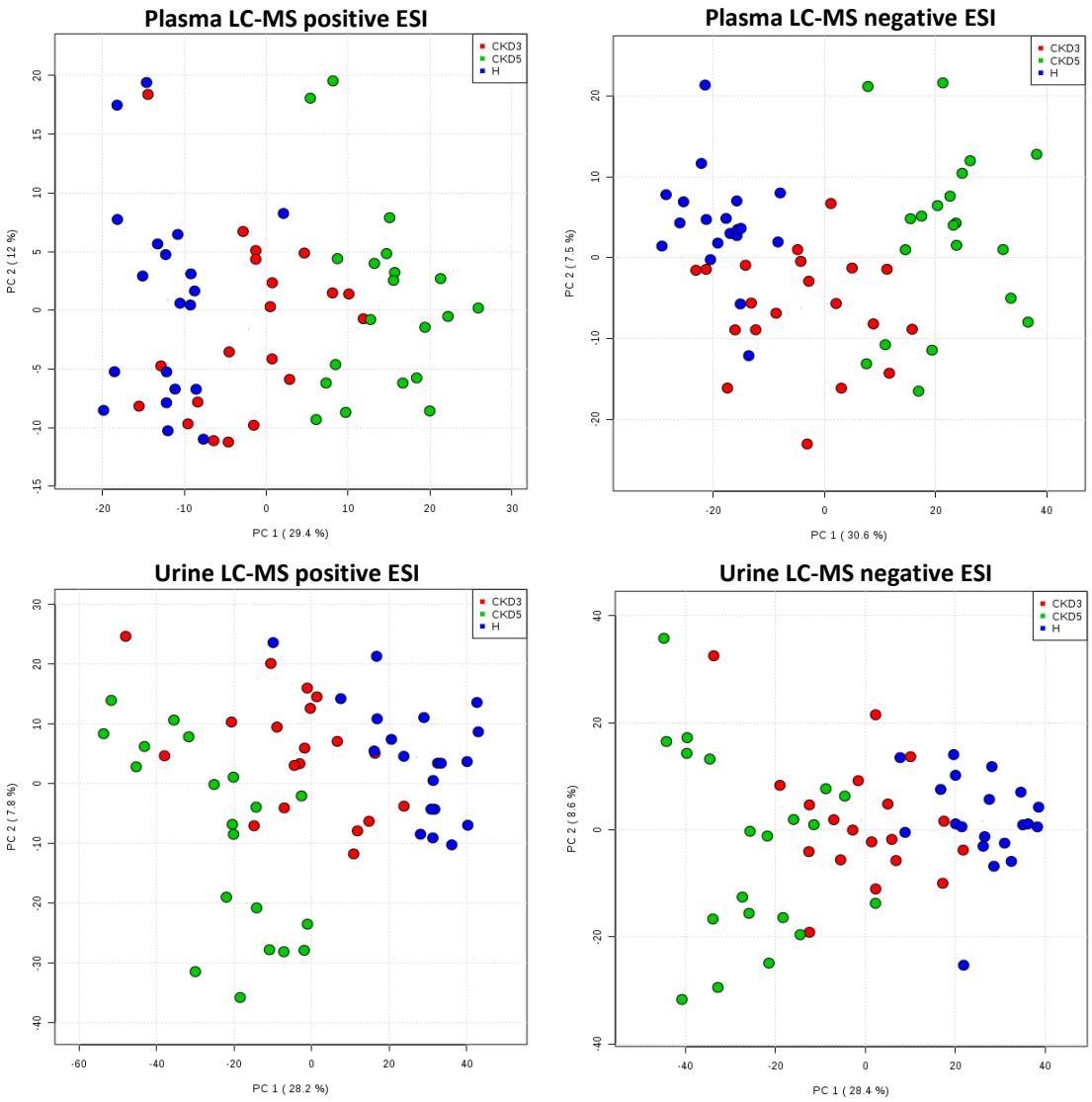
Plasma LC-MS positive ESI
CKD3
CKD5
H
PC 2 (12 %)
PC 1 (29.4 %)
Plasma LC-MS negative ESI
PC 2 (7.5 %)
PC 1 (30.6 %)
Urine LC-MS positive ESI
PC 2 (7.8 %)
PC 1 (28.2 %)
Urine LC-MS negative ESI
PC 2 (8.6 %)
PC 1 (28.4 %)

Fig. 3