New insights in molecular mechanisms involved in chronic kidney disease using high-resolution plasma proteome analysis.

Griet Glorieux[1], William Mullen[2], Flore Duranton[3], Szymon Filip[4,5], Nathalie Gayrard[3], Holger Husi[2], Eva Schepers[1], Nathalie Neirynck[1], Joost P. Schanstra[6, 7], Joachim Jankowski[8], Harald Mischak[2,9], Àngel Argilés[3], Raymond Vanholder[1], Antonia Vlahou[4], Julie Klein[6, 7].*

[1]Nephrology Section, Ghent University Hospital, Gent, Belgium

[2]BHF Glasgow Cardiovascular Research Centre, University of Glasgow, United Kingdom

[3]RD Néphrologie, Montpellier, France

[4]Biomedical Research Foundation, Academy of Athens, Greece

[5]Charité - Universitätsmedizin Berlin, Germany

[6]Institut National de la Santé et de la Recherche Médicale (INSERM), U1048, Institute of Cardiovascular and Metabolic Disease, Toulouse, France

[7]Université Toulouse III Paul-Sabatier, Toulouse, France

[8]University Hospital RWTH, Institute for Molecular Cardiovascular Research, Aachen, Germany

[9]Mosaiques Diagnostics, Hannover, Germany

Corresponding Author

* Julie Klein

Inserm U1048/I2MC

Equipe 12 : Fibrose rénale, mécanismes et détection

1 avenue Jean Poulhès

BP 84225

31432 Toulouse Cedex 4

00 33 5 31 22 40 70

julie.klein64@gmail.com

Abstract

Background: The reduced glomerular filtration rate in the advanced stages of chronic kidney disease (CKD) leads to plasma accumulation of uremic retention solutes including proteins. It has been hypothesized that these changes may, at least in part, be responsible for CKD-associated morbidity and mortality. However, most studies focused on the role of individual proteins while a holistic, large-scale, integrative approach may generate significant additional insight.

Methods: In a discovery study, we analysed the plasma proteomes of patients with stage 2-3 CKD (n=14) and stage 5 CKD with hemodialysis (HD)(n=15), using high-resolution LC-MS/MS analysis. Selected results were validated in cohort of 40 patients with different CKD stages with or without HD, using ELISA.

Results: Of a total of 2054 detected proteins, 127 displayed lower, while 206 displayed higher abundance in plasma of patients on HD. Molecular pathway analysis confirmed modification of known processes involved in CKD complications including decreased hemostasis and increased inflammation, complement activation and vascular damage. In addition, we identified the plasma increase during CKD progression of lysozyme C and leucine-rich alpha-2 glycoprotein, two proteins related to vascular damage and heart failure not yet found to be associated to CKD. Interestingly high level of leucine-rich alpha-2 glycoprotein was associated with higher mortality in stage 5 CKD patients on HD.

Conclusions: This study provides for the first time comprehensive assessment of CKD plasma proteome, contributes to new knowledge and potential markers in the field of late stage CKD and will serve as a basis for future studies investigating the relevance of these molecules in CKD-associated morbidity and mortality.

Keywords: Chronic kidney disease, hemodialysis, uremic solutes, proteomics, mechanisms, biomarkers.

Short summary: Plasma proteome analysis identified >300 proteins with modified abundance in patients on hemodialysis. Systems biology analysis and validation of key-proteins with ELISA suggested the association of these proteins to processes involved in the morbidity and mortality encountered in late stage CKD and identified potential new targets.

Introduction

Chronic kidney disease (CKD) is characterized by progressive loss of renal function, with the final stage being end-stage renal disease (ESRD)[1]. At this stage, patients require permanent renal replacement therapy, *i.e.* hemodialysis (HD)/peritoneal dialysis (PD) or transplantation[1]. As kidney function declines, there is a progressive increase in both mortality and co-morbidities, such as cardiovascular complications [2-6].

The molecular mechanisms involved in CKD and ESRD complications are complex and comprise coagulation abnormalities, endothelial dysfunction, vascular calcification due to impaired calcium and phosphate metabolism, increased oxidative and metabolic stress and inflammation[7-10]. The reduced glomerular filtration rate (GFR) in the advanced stages of CKD affects blood clearance and leads to subsequent accumulation of organic products (uremic retention solutes) and drugs that are normally metabolized or excreted by the kidney. Such uremic retention solutes include proteins, and may induce toxic effects[11, 12]. Significant changes in the blood protein composition have been reported in the past[13-17], and it has been hypothesized that these changes in plasma proteins may, at least in part, be responsible for CKD-associated morbidity and mortality[13, 14, 18]. However these different studies mostly focused on the role of individual proteins and this promising yet disparate molecular evidence indicates a clear need for a holistic, large-scale approach. Plasma proteomics (*i.e.* analysis of the total plasma protein content) could be of particular interest to provide an integrated view and better understanding of CKD-associated complications. The recent advancement in proteomics technologies and software solutions have enabled assessing highly complex samples with high validity[19, 20], indicating that high-resolution plasma proteome analysis should inform about CKD-associated molecular changes.

The rationale of the present study was to apply untargeted high-resolution plasma proteome analysis to investigate molecular changes associated with CKD. Our aim was to identify proteins with altered plasma levels and estimate the functional consequences of such changes.

Material and Methods

Patients

The discovery cohort consisted of 29 patients with CKD. Fourteen CKD patients not treated with HD were recruited from the Department of Nephrology, Transplantation and Dialysis of the University Hospital of Montpellier and the Public Hospital of Sète. Fifteen patients on maintenance HD were recruited from the Néphrologie Dialyse Saint Guilhem Dialysis Unit in Sète. Recruitment was performed between February and June of 2008. The study was approved by the Comité de Protection des Personnes of Montpellier and declared to the French Ministry (reference number DC-2008–417).

The validation cohort consisted out of 32 CKD patients and 8 healthy controls selected from the sample collection of the Nephrology Department of the Ghent University Hospital, Belgium. Subjects were sampled under fasting conditions between January 2011 and July 2012. The study was approved by the local ethical committee (Belgian registration number B67020107926). The study was in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before sampling.

Patients on hemodialysis underwent either hemodialysis or online-hemodiafiltration 3 times 4 hours a week. The quality of the dialysis fluid met the ultrapure standards (bacteria <0.1 CFU/ml, endotoxin <0.03 EU/ml) as checked on a regular basis. Plasma samples were collected

prior to dialysis, before anticoagulant administration. One patient on home hemodialysis was sampled when consulting the out-patient clinic.

K-EDTA-plasma samples were processed immediately after collection and stored at –80°C. Urine protein, C-reactive protein and creatinine concentrations were determined by routine techniques by the hospital laboratory. eGFR was calculated based on serum creatinine using the CKD-EPI creatinine equation. Proteinuria was determined using urine dipstick or with >0.25g protein/g creatinine.

Sample preparation for proteomic analysis

Ten µL of thawed plasma sample was diluted with 90 µL 0.1% sodium dodecyl sulfate, 20mM dithiothreitol, and 0.1 M TrisHCl (pH=7.6). The sample was sonicated at room temperature for 30 minutes, followed by denaturation at 95°C for 3 min. Samples were subsequently incubated with 50 mM Iodoacetamide at room temperature for 30 min in the dark followed by the addition of ammonium bicarbonate buffer solution (300 µL, 50mM), applied to NAP-5 column, and eluted with 1 mL of 50mM ammonium bicarbonate buffer solution.

Twenty µg of lyophilized trypsin was added to 50 µL of activation buffer solution, and 2 µL of this solution was added to the eluted sample. Trypsin digestion was carried out overnight at a temperature of 37°C. Samples were then lyophilized, stored at 4°C and resuspended in 100 µL HPLC-grade H_2O shortly before mass spectrometry analysis.

Proteome analysis

The plasma extracts (5 μl) were analysed by nanoflow LC-MS/MS using a Orbitrap velos FTMS as per[19]. The gradient was run from at 1% B for 5 mins rising to 25% B after 360 mins then on to 65% B after 480 mins.

Proteomics data processing

Peptide analysis was performed using SEQUEST against the Human Uniprot Database as per[19]. The relative quantitative analysis was performed based on the peptide area values. Before analysis, ppm-normalization of peptide areas was conducted.

$$\text{Normalized mass-peak area} = \text{Peptide mass-peak area} / \text{Total sample mass-peak area} \times 10^6$$

Protein abundance was calculated as the sum of all normalized peptide areas for the given protein. Proteins covered by at least two valid peptides were considered valid. Mean protein abundance in the CKD2-3 group was compared to the mean protein abundance in the ESRD/HD group. Protein entries were mapped to the SwissProt database using either the mapping service provided by UniProt or via Blast searching (web.expasy.org/blast/) and merged according to the SwissProt names.

ELISA

Lysozyme C (Abcam (ab108880)), Complement factor D (R&D Systems (DFD00)), Leucine-rich alpha-2-glycoprotein (Cusabio (CSB-E12962h)) and histidine-rich glycoprotein (Cusabio (CSB-E13159h)) were quantified by ELISA according to manufacturer's guidelines. Samples were analysed using the EL808 Ultra Microplate Reader from Bio-Tek Instruments (Winooski, VT) using KC4V3.0 Analysis Software.

Functional analysis

Functional analysis was performed with Ingenuity Pathway Analysis (Qiagen) and Cytoscape (version 3.0.2; ClueGO plugin version 2.0.6) software. Individual analyses were performed by comparison of up-regulated versus down-regulated proteins, thereby eliminating common terminology-clusters associated with both conditions, using the GO-class of biological functions.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software. F-test was performed to test for data distribution. When data were normally distributed, parametric t-test was performed; otherwise, the statistical analysis was performed using Mann-Whitney test or Wilcoxon signed rank test. Multiple hypotheses testing correction was performed using Benjamini-Hochberg test for false discovery rate. Comparison of survival curves was performed using Log-rank (Mantel-Cox) test.

Results

Plasma proteome analysis

We studied the plasma proteome of 14 patients with moderate CKD (stage 2 and 3, CKD2-3 group) and 15 patients on hemodialysis for at least one year (CKD5/HD group) (Table 1). A schematic of the design is given in Figure 1. LC-MS/MS analysis enabled identification of 9017 peptides (Supplementary Table 1), representing a total of 2054 unique proteins quantified using a label-free approach (Supplementary Table 2). To assess the validity of the quantification strategy, the reported concentration of 20 high abundant plasma proteins[21-23] (Supplementary Table 3) was compared to their respective relative abundance in the LC-MS/MS experiment

(normalized-ppm mass-peak area, arbitrary units). As evident from Figure 2A significant correlation between existing data and LC-MS/MS quantification was observed (CKD2-3 Spearman r = 0.8480, p<0.0001, CKD5/HD Spearman r = 0.7889, p<0.0001). A further validation of the plasma proteome-based approach was obtained by measuring C-reactive protein (CRP). We observed significant correlation between quantitative measurement of CRP obtained by routine laboratory testing (Table 1) and semi-quantitative LC-MS/MS abundance in the discovery cohort (Figure 2B), confirming the validity of the approach.

Plasma proteome changes in CKD5/HD versus CKD2-3 patients

When comparing the 15 datasets from patients on HD with the 14 from patients at early stage CKD, we identified 333 proteins as significantly different between the two groups, 127 with lower and 206 with higher abundance in CKD5/HD compared to CKD2-3 (Supplementary Table 2). Upon correction for multiple testing, 39 of the 333 remained significant (Table 2). To exclude bias introduced by age or gender, we also investigated an age- and sex-matched subcohort of 7 CKD2-3 patients and 7 CKD5/HD patients (Table 1). Although this subcohort comparison is of lower statistical power, the initial results could be generally confirmed (Table 2).

Using LC-MS/MS, we confirmed increased abundance of 7 of 39 well-characterized uremic toxins[11, 12] including beta-2-microglobulin and prostaglandin-H2 D-isomerase, while two of them were detected but showed unmodified abundance (i.e. immunoglobulins kappa and lambda) (Supplementary Table 4).

Pathway analysis

Functional analysis of the 333 modified plasma proteins was performed using Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) software. The pathways with the highest modification scores included acute phase response signaling and complement and coagulation systems (Figure 3-4, Supplementary Table 5). Other pathways included platelet degranulation, calcium ion-dependent exocytosis, response to selenium ion, liver X receptor (LXR)/retinoid R receptor (RXR) activation, atherosclerosis signaling and production of nitric oxide (NO) and reactive oxygen species (ROS) (Supplementary Table 5).

Of the 33 proteins associated with the complement system, 24 were detected in the plasma using LC-MS/MS. Among those 5 were significantly increased in abundance in the plasma of patients at CKD5/HD compared to CKD2-3 (Figure 3A), suggesting activation of the complement system in advanced CKD. On the other hand, 23 out of 35 proteins associated with the coagulation system were detected in the plasma using LC-MS/MS (not shown), and levels of 5 were significantly modified, 3 with lower, and 2 with higher abundance in CKD5/HD compared to CKD2-3 (Figure 3B). From a total of 169 proteins associated with acute phase response signaling in Ingenuity database, 62 were detected in plasma and 17 were significantly modified in CKD5/HD patients (Figure 4). Most of these changes were in accordance with the activation of the pathway (Figure 4): 9 proteins known to be increased in the plasma during the acute phase response were found to be more abundant, and 4 known to be decreased were found to be less abundant in CKD5/HD compared to CKD2-3 (Figure 4). In particular, CRP levels determined by LC-MS/MS or routine analysis showed similar increase in the plasma of CKD5/HD patients compared to CKD2-3 (Figure 4, Bold and Table 1), confirming activation of the acute phase response in CKD. Of note, the same analysis was performed after exclusion of 6 patients of the CKD5/HD group with highest CRP values and this lead to same results.

Validation of newly identified plasma proteins using ELISA

We next aimed to validate key novel proteins in an independent cohort of healthy controls and patients with stage 3b (CKD3b), stage 4 (CKD4), and stage 5 CKD not on HD (CKD5 no HD) and on HD (CKD5/HD)(n=8/group), all matched for age and gender (Table 1) using ELISA. We confirmed that the abundance of complement factor D, lysozyme C and leucine-rich alpha-2 glycoprotein were significantly increased in plasma of CKD5 patients compared to controls. We further showed that their abundance progressively increased during CKD progression (Figure 5A-5C). We also demonstrated that protein levels were increased in the CKD5/HD group compared to CKD5 not on dialysis (Figure 5E-5G). We could not validate LC-MS/MS change for histidine-rich glycoprotein (Figure 5D and 5H). We further correlated plasma concentrations of the proteins with the eGFR of the patients. Complement factor D ($r=-0.9084$, $p<0.0001$), lysozyme C ($r=-0.7115$, $p<0.0001$) and leucine-rich alpha-2 glycoprotein ($r=-0.8236$, $p<0.0001$) were inversely correlated with eGFR while histidine-rich glycoprotein showed no significant correlation ($r=-0.0046$, n.s.)(Supplementary Figure).

Association of complement factor D, lysozyme C, leucine-rich alpha-2 glycoprotein and histidine-rich glycoprotein to mortality in CKD5/HD patients

We next assessed the association of complement factor D, lysozyme C, leucine-rich alpha-2 glycoprotein and histidine-rich glycoprotein to the occurrence of all-cause mortality in CKD patients after a follow-up period of 3.14±0.14 years post sampling (Figure 6). In the discovery cohort, no mortality was observed in the CKD2-3 patient group. From the 15 patients

of the CKD5/HD group, two patients were excluded as one was transplanted and one was lost for follow-up. Among the 13 remaining patients, 10 died and 3 were still alive at the last follow-up. We determined the median LC-MS/MS abundance as the threshold for the four proteins. We observed that high abundance of leucine-rich alpha-2 glycoprotein was significantly associated with higher mortality in patients with CKD5 on hemodialysis compared to patients with low leucine-rich alpha-2 glycoprotein abundance (Figure 6C). Level of leucine-rich alpha-2 glycoprotein was neither affected by age (73.86±4.37 and 71.33±6.15 years old in low versus high, p>0,05) nor time on dialysis (2±1.26 and 2±1.15 years in low versus high, p>0.05).

Discussion

The aim of the present study was to assess global plasma protein changes in late stage CKD patients and identify those proteins associated to CKD progression. The knowledge provided by this global approach is likely to help in progressing in our understanding of the pathophysiology of the different aspects of CKD and in recognizing those of prognosis value. To this aim, we compared the plasma proteome changes in patients with moderate CKD (stage 2 and 3) to patients with advanced CKD undergoing HD using LC-MS/MS (Figure 1).

Uremic toxins are solutes normally excreted by the kidneys, which accumulate and negatively impact biologic functions when renal function declines. Reviews from the European Uremic Toxin Work Group listed and classified 39 uremic retention proteins and their normal and uremic concentrations, as measured with antibody-based techniques, such as ELISA or radioimmunoassay[11, 12]. In our study, we confirmed the accumulation of seven of these well-known uremic solutes (*e.g.* beta-2-microglobulina and prostaglandin-H2 D-isomerase) out of the 333 proteins that were significantly different between the two groups. The technology did not

allow the detection of the other uremic toxins most likely due to their very low molecular weight (e.g. proenkephalin-A) and/or their very low abundance (e.g. fibroblast growth factor 23) and the masking effect of highly abundant plasma proteins such as albumin (Supplementary Table 4).

To verify our findings, we focused on new candidates generally not described in the context of CKD (lysozyme C, leucine-rich alpha-2-glycoprotein and histidine-rich glycoprotein). Using ELISA, we observed in an independent validation cohort that lysozyme C and leucine-rich alpha-2-glycoprotein abundance was increased in CKD patients compared to healthy controls, and were further increased during HD (Figure 5). Conversely, although histidine-rich glycoprotein level was clearly significantly decreased in ESRD as measured using LC-MS/MS, we were not able to confirm this difference using ELISA. Such technical discrepancy between MS-based and antibody-based approaches such as western blot or ELISA is not uncommon and has been observed in other proteome studies[24-26], making the validation process, yet necessary, sometimes difficult.

Circulating lysozyme C, a protein released from leukocytes and macrophages and primarily known for its bacteriolytic function, binds to the endocardial endothelium and within the vascular smooth muscle layer of arteries, leading to the generation of hydrogen peroxide and nitric oxide and ultimately causing persistent systemic vasodilatation and myocardial depression [27, 28]. Leucine-rich alpha-2-glycoprotein is a secreted glycoprotein of not too well known function and has been linked to different type of cancer, appendicitis and ulcerative colitis[29-34]. It also promotes aberrant angiogenesis[35] which is a key feature of a number of diseases including age-related macular degeneration, diabetic retinopathy, cancer, atherosclerosis and plaque rupture[36]. Moreover, leucine rich alpha-2 glycoprotein was previously shown to be excreted in the urine of patients with IgA nephropathy and CKD[37, 38]. Plasma accumulation of the proteins

was significantly correlated with renal function decline (Supplementary Figure), showing that the increased plasma levels of the molecules reflect at least in part alteration of glomerular filtration. Although we do not know whether this accumulation can be responsible for the development of complications in patients with advanced CKD, it is interesting to note that increased plasma level of lysozyme C was significantly associated with atheromatous disease and could predict the severity of coronary artery disease[39], while increased serum leucine-rich alpha-2-glycoprotein has been found to be a biomarker of heart failure[29].

The functional relevance of the 333 proteins identified with our proteomic approach and their putative prognostic value, are to be further explored. As a first attempt to gain inside we assessed the cellular activities to which these proteins are related with and observed that many of them participate in acute phase response and complement and coagulation system activation (Figure 3-4, Supplementary Table 5). Activated systemic inflammatory state is a very well-known epiphenomenon of advanced CKD and dialysis[40, 41]. The levels of CRP and other acute-phase reactants proteins such as alpha-1-antichymotrypsin (SerpinA3) and alpha-1-acid glycoprotein (Orm1) were increased in our patients with ESRD and HD confirming acute inflammatory response in uremic conditions (Figure 4 and Supplementary Table 5). Moreover, patients with advanced CKD displayed elevated levels of different members of the complement cascade, such as complement components C1r, C1s, C4, C9 and complement factor D. We confirmed in the validation cohort that complement factor D abundance was increased in CKD patients compared to healthy controls, and was further increased in patients undergoing chronic HD (Figure 5). Complement factor D is a serine protease catabolized by the kidney, resulting in increased circulating complement factor D concentrations in patients with chronic renal failure and on long-term dialysis[42]. The question remains whether plasma accumulation of complement

factor D is only the reflection of an alteration of kidney filtration or is an actor involved in the increased risk of CKD associated complications. Although data are scarce, it has been suggested that complement factor D might be involved in the development of vascular smooth muscle cells abnormalities and arterial cartilaginous lesions in a mouse model of vascular calcifications[43, 44]. In light of our results, further studies should be performed in order to better understand the origin and the role complement factor D and complement system in patients with CKD and HD and its link to vascular disease.

In addition to these inflammatory risk factors, changes in the expression of coagulation factors F5, F10 and F12, as well as in plasma kallikrein (Klkb1), proteins related to blood coagulation and platelet activation (Figure 3B and Supplementary Table 5) were observed. Changes in hemostasis are well described in CKD[41] due to uremic toxins, interaction with dialyzer membranes and the repeated use of anticoagulants all together contributing to the risk of cardiovascular and thrombotic complications in patients undergoing HD[41]. In 1996, Tan et al analyzed the plasma activity of different coagulation factors in patients with IgA nephropathy and found that IgA patients had significantly lower plasma activity of F12 and Klkb1 compared to healthy controls, which was consistent with our observations[45]. More recently, the plasma activity of F5 was found significantly higher in diabetic patients with renal disease compared with diabetic patients without renal disease[46], a finding in accordance with the observed increased level of F5 in the plasma of CKD patients. However, in this study, plasma activity of F10 tended to decrease as observed in our study but this not reached significant level[46].

One of the main limitations of this study is that observed molecular changes have not been related to clinical endpoints such as infections, hemorrhages, atherosclerosis or cardiovascular events except for leucine-rich alpha-2 glycoprotein for which we observed that

high plasma levels are significantly associated with higher mortality in patients with CKD5 on hemodialysis compared to patients with low leucine-rich alpha-2 glycoprotein abundance (Figure 6C). Indeed, this study was designed as a discovery study to first identify new candidate proteins among a broad array of hundreds of potential candidates with significant plasma changes, which may in subsequent studies enable better understanding of HD-associated complications.

In conclusion, in this study we have generated for the first time a comprehensive assessment of the plasma proteome of ESRD patients in comparison to early stage CKD, in a cohort of significant size that enabled identification of a large array of proteins altered in ESRD, using high-resolution LC-MS/MS analysis. This study serves as a basis for future studies investigating the relevance of the observed changes, but also as a well-defined comparator for similar studies in the future. In addition, the data may serve for further biomarker identification and systems biology-based assessment of the molecular mechanisms involved CKD-associated morbidity and mortality.

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Conflict of interest

None. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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Table 1. Patient selection. The table summarizes the demographic and clinical data of the patients included in the study. n.d.: not detected. n.s: not significant. n.a.: not available.

	Discovery cohort (complete)			Discovery sub cohort (matched)			Validation cohort					
	CKD2-3	**CKD5/HD**	**pValue**	**CKD2-3**	**CKD5/HD**	**pValue**	**Controls**	**CKD3b**	**CKD4**	**CKD5 no HD**	**CKD5/HD**	**pValue**
N	14	15		7	7		8	8	8	8	8	
Male/Female	6/8	12/3	0.039	3/4	4/3	n.s.	4/4	4/4	4/4	4/4	4/4	n.s.
eGFR (mL/min/1.73m²)	63.78 ±14.42	9.13 ± 3.20	<0.0001	61.30 ±12.39	<10	0.0006	-	37.05 ±4.00	21.81 ±5.46	12.17 ±2.21	<10	<0.0001
Proteinuria (Yes/No)	2/12	n.a.		0/7	n.a.		n.a.	5/3	3/3 (2 n.a.)	5/1 (2 n.a.)	n.a.	
Age (years)	57.16 ±11.63	70.33 ±8.49	0.004	64.51 ±8.57	65.45 ±9.67	n.s.	50.64 ±13.25	60.08 ±10.33	57.92 ±14.66	63.17 ±20.48	59.67 ±10.46	n.s.
Time on dialysis (year)	-	2.58 ±2.57		-	3.00 ±3.21		-	-	-	-	5.17 ±4.32	
BMI (kg/m²)	29.39 ±6.79	25.81 ±4.78	n.s.	30.99 ±6.99	26.63 ±6.22	n.s.	23.97 ±4.62	26.88 ±3.96	26.87 ±4.01	27.10 ±5.13	29.55 ±7,98	n.s.
Systolic blood pressure (mmHg)	134±15	150±19	0.032	144±14	154±10	n.s.	124±14	143±21	139±19	128±10	143±16	0.017
Diastolic blood pressure (mmHg)	71±8	77 ±9	n.s.	74±8	78±10	n.s.	75 ±9	82 ±9	84 ±12	80 ±6	71 ±16	n.s.
C-Reactive Protein (mg/L)	2.71 ±2.71	13.35 ±14.26	0.003	3.43 ±3.14	16.46 ±15.72	n.s.	2.22 ±1.52	2.75 ±2.22	3.00 ±1.21	3.25 ±4.75	6.2 ±6.12	n.s.
Etiologies												
Diabetes	4	7	n.s.	3	4	n.s.	-	2	1	1	2	n.s. (between all groups)
Vascular	4	3		4	2		-	0	3	1	2	
Glomerular	1	2		0	0		-	2	1	2	0	
ADPKD	0	0		0	0		-	2	0	2	2	
Other/Unknown	5	3		0	1		-	2	3	2	2	
Medications (%)												
Vitamin K anta	7	7	n.s.	14	0	n.s.	0	25	13	0	0	n.s.
Antiaggregant	0	53	0.0022	0	43	n.s.	0	63	63	50	25	0.044
ACEi/ARB	36	33	n.s.	43	29	n.s.	25	75	63	88	50	n.s.

Statin	21	33	n.s.	29	29	n.s.	13	50	50	88	50	n.s.
Cortisone	0	0	n.s.	0	0	n.s.	0	0	0	0	0	n.s.

Table 2. Plasma proteome changes in CKD2-3 versus CKD5/HD patients. List of 39 modified proteins identified with LC-MS/MS in the complete discovery cohort (adjusted pValue<0.05) and in the age- and sex- matched sub cohort. Bold: protein validated with ELISA in the validation cohort. n.d.: not detected. n.s.: not significant.

			Discovery cohort (complete)				Discovery sub cohort (matched)		
Symbol	**Name**	**Molecular weight (kDa)**	**Fold change**	**Direction**	**Unadjusted pValue**	**Adjusted pValue**	**Fold change**	**Direction**	**Unadjusted pValue**
IGFBP6	Insulin-like growth factor-binding protein 6	23	n.d. in CKD2-3	Up	0,0001	0,0128	n.d. in CKD2-3	Up	0,0156
LYZ	Lysozyme C	15	n.d. in CKD2-3	Up	0,0002	0,0187	n.d. in CKD2-3	Up	0,0156
B2M	Beta-2-microglobulin	12	99,03	Up	0,0000	0,0105	68,3	Up	0,0170
CFD	Complement factor D	24	97,8	Up	0,0000	0,0044	n.d. in CKD2-3	Up	0,0156
PTGDS	Prostaglandin-H2 D-isomerase	19	32,36	Up	0,0003	0,0245	37,36	Up	0,0364
PRPF3	U4/U6 small nuclear ribonucleoprotein Prp3	78	28,16	Up	0,0001	0,0150	20,4	Up	0,0265
WDFY4	WD repeat- and FYVE domain-containing protein 4	354	7,82	Up	0,0002	0,0204	18,77	Up	0,0088
RUSC2	Iporin	161	6,02	Up	0,0000	0,0012	16,41	Up	0,0003
C1S	Complement C1s subcomponent	47 & 28	4,54	Up	0,0001	0,0128	3,7	Up	0,0018
SSX2IP	Afadin- and alpha-actinin-binding protein	71	4,53	Up	0,0001	0,0114	4,04	Up	n.s.
LENG8	Leukocyte receptor cluster member 8	86	4,41	Up	0,0000	0,0044	4,17	Up	0,0009
AMBP	Protein AMBP	21 & 16 & 7	3,98	Up	0,0000	0,0039	4,05	Up	0,0004
CCDC147	Coiled-coil domain-containing protein 147	103	3,57	Up	0,0002	0,0180	6,53	Up	0,0121
SETD2	Histone-lysine N-methyltransferase SETD2	288	3,37	Up	0,0009	0,0499	3,21	Up	0,0282
NR0B1	Nuclear receptor subfamily 0 group B member 1	52	3,15	Up	0,0001	0,0128	5,18	Up	0,0007

C1R	Complement C1r sucomponent	51 & 27	3	Up	0,0001	0,0123	2,46	Up	0,0271
HBB	Hemoglobin subunit beta	16	2,85	Up	0,0006	0,0403	5,53	Up	0,0036
CNNM4	Metal transporter CNNM4	87	2,55	Up	0,0002	0,0204	1,97	Up	n.s.
SLC9A5	Sodium/hydrogen exchanger 5	99	2,23	Up	0,0002	0,0167	2,69	Up	0,0028
TFAM	Transcription factor A, mitochondrial	24	2,15	Up	0,0007	0,0427	2,28	Up	0,0094
ITPR1	Inositol 1,4,5-trisphosphate receptor type 1	314	2,01	Up	0,0006	0,0394	1,75	Up	0,0133
SERPINF1	Pigment epithelium-derived factor	44	1,92	Up	0,0003	0,0228	2,63	Up	0,0037
IGHA1	Ig alpha-1 chain C region	38	1,88	Up	0,0006	0,0403	1,66	Up	0,0096
CSPP1	Centrosome and spindle pole-associated protein 1	146	0,64	Down	0,0008	0,0459	0,77	Down	n.s.
LRRIQ3	Leucine-rich repeat and IQ domain-containing protein 3	74	0,6	Down	0,0003	0,0210	0,62	Down	0,0194
FNBP1	Formin-binding protein 1	71	0,54	Down	0,0001	0,0134	0,54	Down	0,0025
RLTPR	Leucine-rich repeat-containing protein 16C	155	0,5	Down	0,0003	0,0242	0,56	Down	0,0283
HRG	Histidine-rich glycoprotein	58	0,47	Down	0,0006	0,0403	0,39	Down	0,0153
F12	Coagulation factor XII	40 & 26	0,46	Down	0,0009	0,0472	0,45	Down	0,0306
ARHGAP25	Rho GTPase-activating protein 25	73	0,42	Down	0,0008	0,0472	0,7	Down	n.s.
SNIP1	Smad nuclear-interacting protein 1	46	0,37	Down	0,0002	0,0179	0,42	Down	0,0281
ZNF415	Zinc finger protein 415	69	0,3	Down	0,0007	0,0427	0,13	Down	0,0018
CALR3	Calreticulin-3	43	0,29	Down	0,0009	0,0499	0,24	Down	0,0135
ZFAND4	AN1-type zinc finger protein 4	80	0,23	Down	0,0000	0,0014	0,28	Down	0,0087
NOS2	Nitric oxide synthase, inducible	131	0,16	Down	0,0000	0,0044	0,31	Down	0,0255
GPX3	Glutathione peroxidase 3	23	0,15	Down	0,0000	0,0012	0,13	Down	0,0021
PDE4B	cAMP-specific 3,5-cyclic phosphodiesterase 4B	83	0,15	Down	0,0001	0,0128	0,07	Down	0,0009
SBF1	Myotubularin-related protein 5	208	0,05	Down	0,0000	0,0044	0,08	Down	0,0042

MIPEP	Mitochondrial peptidase	intermediate	77	0,01	Down	0,0001	0,0105	0,01	Down	0,0421	

Figure legends

Figure 1. Overview of the study design.

Figure 2. Correlation of quantification of plasma proteins identified using LC-MS/MS with their plasma concentrations. A. Correlation of the published concentrations of the 20 most abundant plasma proteins as measured in the standard plasma using immune assays to their relative LC-MS/MS abundance in the plasma of the CKD2-3 or CKD5/HD patients as quantified in our study using the normalized-ppm mass-peak area (arbitrary units). **B.** Correlation of the absolute concentration of C-reactive protein (CRP) to its relative LC-MS/MS abundance in the plasma of the CKD2-3 or CKD5/HD patients.

Figure 3. Alteration of the complement and coagulation systems. Simplified view of the complement (**A**) and coagulation (**B**) systems using Ingenuity pathway designer. Grey: not modified; Green: decreased abundance; Red: increased abundance; White: not detected with LC-MS/MS; Oval shape: pathway-specific proteins; Square shape: proteins shared with different pathways.

Figure 4. Alteration of the acute phase response pathway. Simplified view of the acute phase response signaling pathway using Ingenuity pathway designer. Grey: not modified; Green: decreased abundance; Red: increased abundance; White: not detected with LC-MS/MS; Oval shape: pathway-specific proteins; Square shape: proteins shared with different pathways.

Figure 5. Validation of plasma protein modifications during CKD progression and HD. Plasma changes of complement factor D **(A, E)**, lysozyme C **(B, F)**, leucine-rich alpha-2-glycoprotein **(C, G)**, and histidine-rich glycoprotein **(D, H)** identified with high confidence using LC-MS/MS were confirmed using ELISA in an independent validation cohort of healthy controls (n=8), and patients with CKD3b (n=8), CKD4 (n=8), CKD5 not in HD (CKD5 no HD, n=8) and CKD5 with HD (CKD5/HD, n=8). *$p<0.05$; ****$p<0.0001$.

Figure 6. Association of plasma protein levels with mortality in CKD5/HD patients. Survival analysis was performed on CKD5/HD patients from the discovery cohort stratified based on high (solid line) versus low (dashed line) LC-MS/MS abundance of complement factor

D (High n=6; Low n=7) (**A**), lysozyme C (High n=6; Low n=7) (**B**), leucine-rich alpha-2-glycoprotein (High n=6; Low n=7) (**C**) and histidin-rich glycoprotein (High n=6; Low n=7) (**D**).

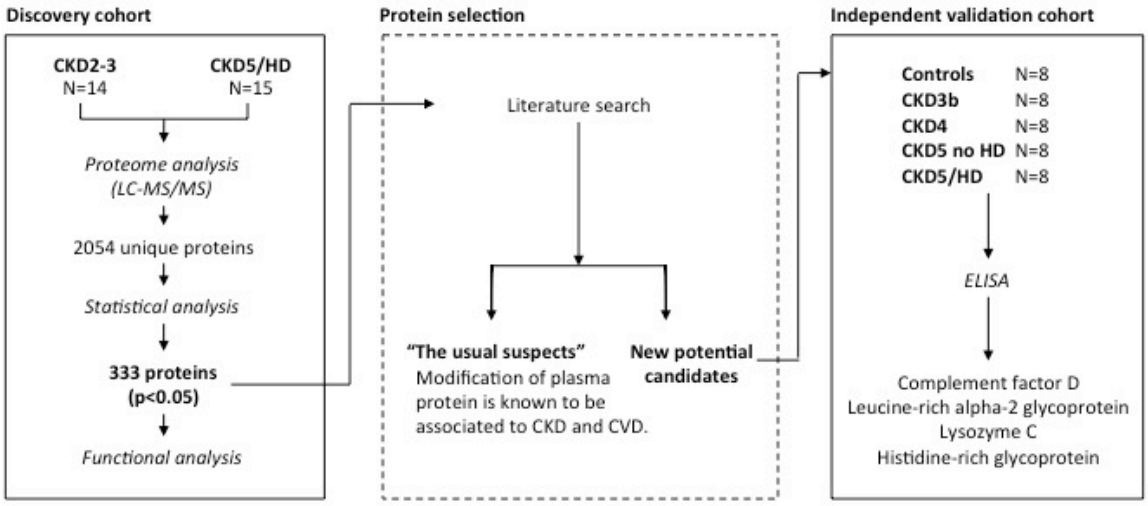

Figure 1

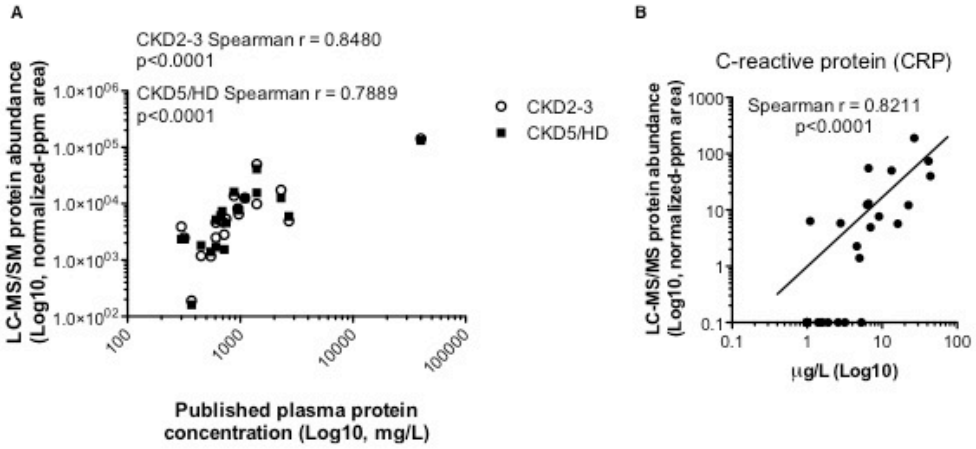
A
CKD2-3 Spearman r = 0.8480
p<0.0001
CKD5/HD Spearman r = 0.7889
p<0.0001
CKD2-3
CKD5/HD
LC-MS/SM protein abundance
(Log10, normalized-ppm area)
Published plasma protein
concentration (Log10, mg/L)
B
C-reactive protein (CRP)
Spearman r = 0.8211
p<0.0001
LC-MS/MS protein abundance
(Log10, normalized-ppm area)
µg/L (Log10)

Figure 2

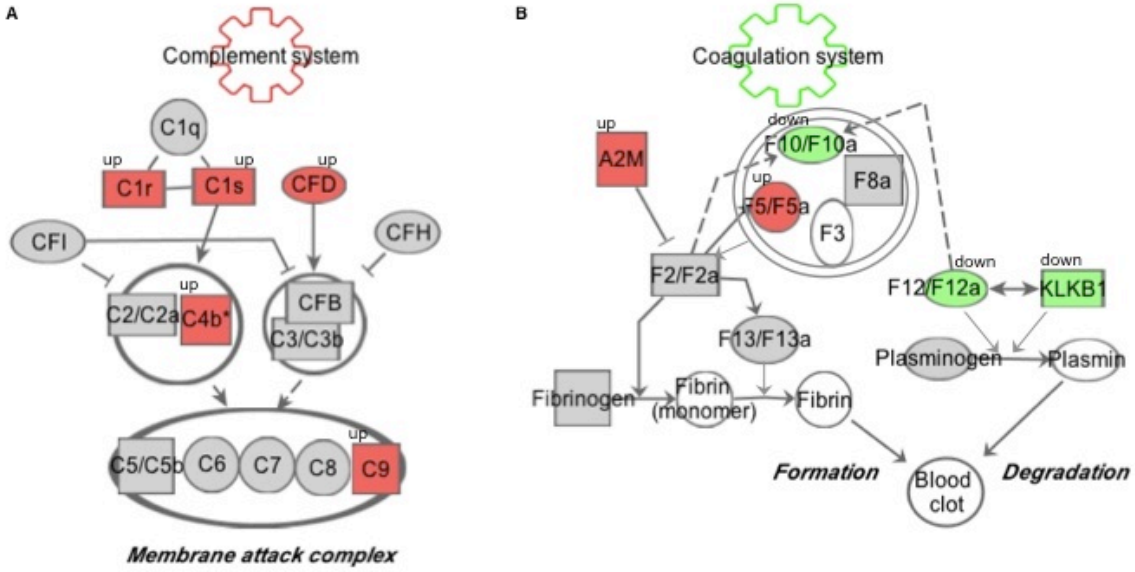
A
Complement system
C1q
up
C1r
up
C1s
up
CFD
CFI
CFH
up
C2/C2a
C4b*
CFB
C3/C3b
up
C5/C5b
C6
C7
C8
C9
Membrane attack complex
B
Coagulation system
up
A2M
down
F10/F10a
F8a
up
F5/F5a
F3
F2/F2a
down
F12/F12a
down
KLKB1
F13/F13a
Plasminogen
Plasmin
Fibrinogen
Fibrin
(monomer)
Fibrin
Formation
Blood
clot
Degradation

Figure 3

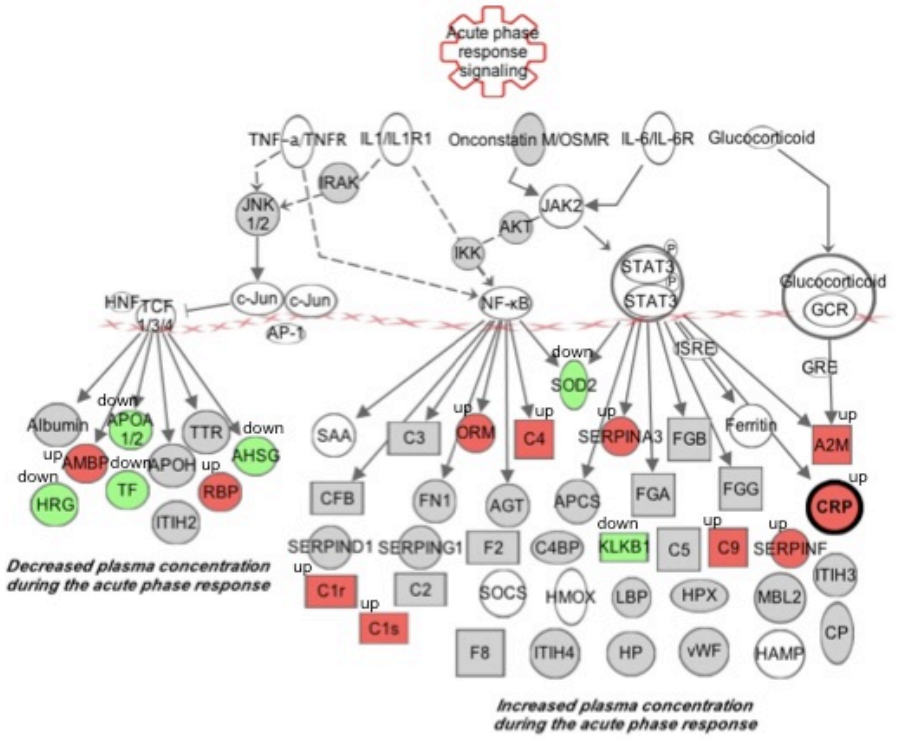
Acute phase response signaling
TNF-a/TNFR
IL1/IL1R1
Onconstatin M/OSMR
IL-6/IL-6R
Glucocorticoid
IRAK
JNK 1/2
JAK2
AKT
IKK
STAT3
STAT3
Glucocorticoid
GCR
HNF
TCF 1/3/4
c-Jun
c-Jun
AP-1
NF-κB
down
SOD2
ISRE
GRE
down
Albumin
APOA 1/2
TTR
down
AHSG
up
AMBP
down
APOH
up
down
HRG
TF
RBP
ITIH2
SAA
C3
up
ORM
up
C4
up
SERPINA3
FGB
Ferritin
up
A2M
up
CRP
CFB
FN1
AGT
APCS
FGA
FGG
down
KLKB1
up
C9
up
SERPINF
SERPIND1
SERPING1
F2
C4BP
C5
ITIH3
up
C1r
C2
SOCS
HMOX
LBP
HPX
MBL2
up
C1s
CP
F8
ITIH4
HP
vWF
HAMP
Decreased plasma concentration during the acute phase response
Increased plasma concentration during the acute phase response

Figure 4

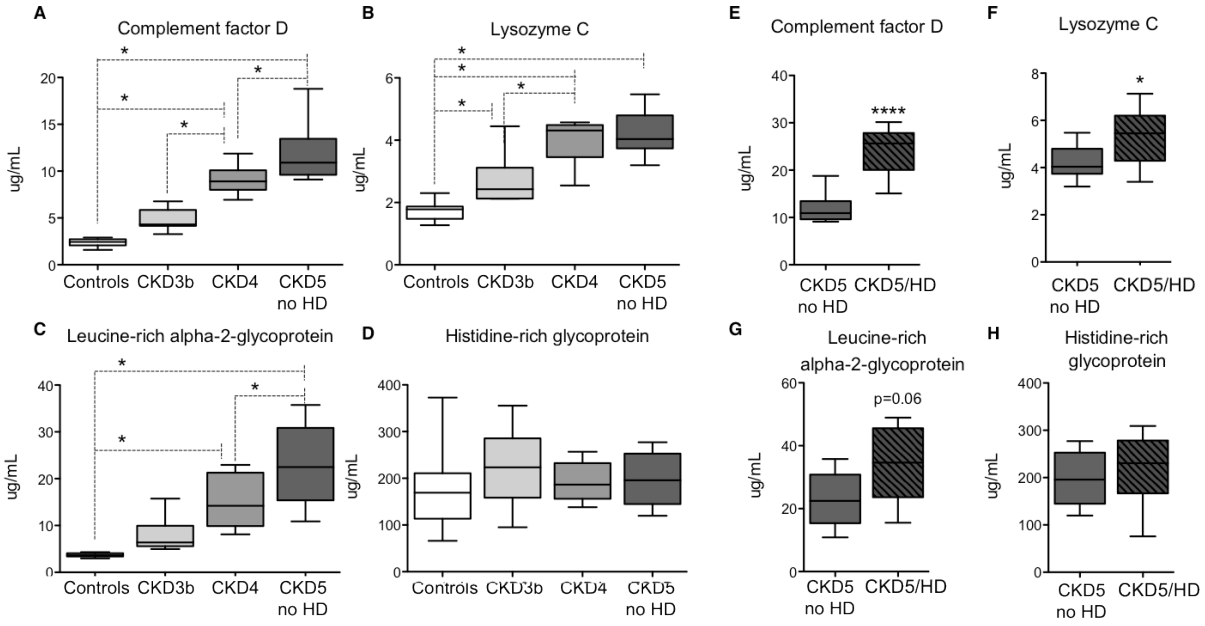
A
Complement factor D
ug/mL
Controls
CKD3b
CKD4
CKD5 no HD
B
Lysozyme C
ug/mL
Controls
CKD3b
CKD4
CKD5 no HD
E
Complement factor D
ug/mL
****
CKD5 no HD
CKD5/HD
F
Lysozyme C
ug/mL
*
CKD5 no HD
CKD5/HD
C
Leucine-rich alpha-2-glycoprotein
ug/mL
Controls
CKD3b
CKD4
CKD5 no HD
D
Histidine-rich glycoprotein
ug/mL
Controls
CKD3b
CKD4
CKD5 no HD
G
Leucine-rich alpha-2-glycoprotein
p=0.06
ug/mL
CKD5 no HD
CKD5/HD
H
Histidine-rich glycoprotein
ug/mL
CKD5 no HD
CKD5/HD

Figure 5

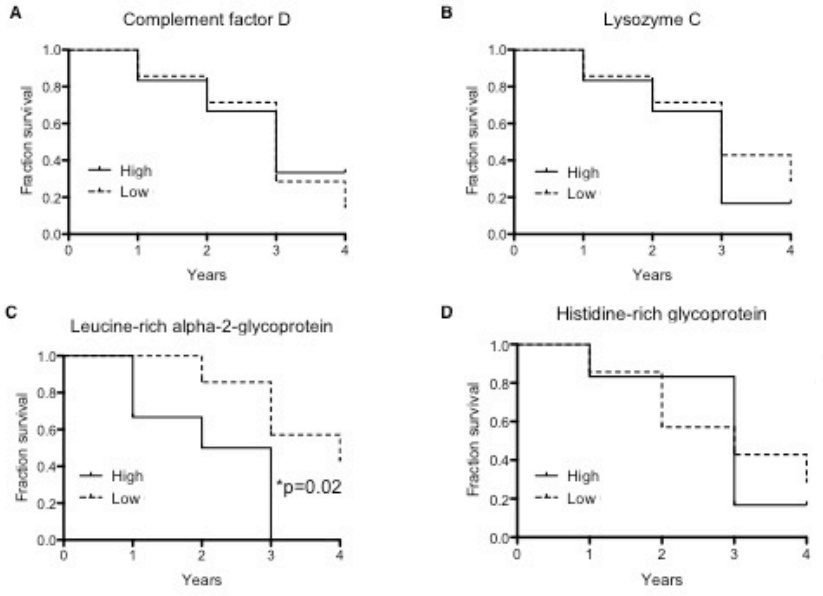

Figure 6

Supporting Information.

Supplementary Figure. Correlation of plasma protein concentrations to eGFR. Plasma concentrations of complement factor D, lysozyme C, leucine-rich alpha-2-glycoprotein and histidine-rich glycoprotein using ELISA was correlated to eGFR in the independent validation cohort.

Supplementary Table 1. Peptide identification using LC-MS/MS. The table displays the complete list of 9017 tryptic peptides identified using LC-MS/MS associated with their relative abundance (expressed using normalized-ppm mass-peak area (arbitrary units)) and their regulation in the CKD5/HD group compared with CKD2-3 group.

Supplementary Table 2. Protein identification using LC-MS/MS. The table displays the complete list of 2054 proteins identified using LC-MS/MS by combination of tryptic peptides, their relative abundance (expressed in ppm) and their regulation in the CKD5/HD group compared with CKD2-3 group.

Supplementary Table 3. Highly abundant plasma proteins. The table displays the list of the 20 most abundant plasma proteins associated with their respective published concentrations as measured in the standard plasma using immune assays and their relative LC-MS/MS abundance in the plasma of the CKD2-3 or CKD5/HD patients as quantified in our study using the normalized-ppm mass-peak area (arbitrary units).

Supplementary Table 4. LC-MS/MS detection of uremic toxins. The table displays the 25 known low-molecular weight proteins that accumulate in uremic syndrome, their reported normal and uremic concentrations as reviewed in Duranton et al., 2012 (PMID: 22626821) and Vanholder et al., 2003 (PMID: 12675874) and their detection using LC-MS/MS.

Supplementary Table 5. Functional analysis of plasma proteome changes. List of functional pathways modified using Gene Ontology (GO) and Ingenuity Pathway Analysis software (IPA) associated with significantly modified proteins. Bold: proteins with adjusted pValue<0.05.