Nephrol Dial Transplant (2015) 30: 1842–1852
doi: 10.1093/ndt/gfv254
Advance Access publication 9 July 2015

ndt
Nephrology Dialysis Transplantation

Original Article

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[4,10] and Julie Klein[6,7]

[1]Nephrology Section, Ghent University Hospital, Gent, Belgium, [2]BHF Glasgow Cardiovascular Research Centre, University of Glasgow, Glasgow, UK, [3]RD Néphrologie, Montpellier, France, [4]Biomedical Research Foundation, Academy of Athens, Athens, Greece, [5]Charité - Universitätsmedizin Berlin, 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 and [10]School of Biomedical & Healthcare Sciences, Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth, UK

Correspondence and offprint requests to: Julie Klein; E-mail: julie.klein@inserm.fr

ABSTRACT

Background. The reduced glomerular filtration rate in the advanced stages of chronic kidney disease (CKD) leads to plasma accumulation of uraemic 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 proteome of patients with stage 2–3 CKD ($n = 14$) and stage 5 CKD with haemodialysis (HD) ($n = 15$), using high-resolution LC–MS/MS analysis. Selected results were validated in a 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 the plasma of patients on HD. Molecular pathway analysis confirmed the modification of known processes involved in CKD complications, including decreased haemostasis 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. 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 a comprehensive assessment of CKD plasma proteome, contributing to new knowledge and potential markers of CKD. These results will serve as a basis for future studies investigating the relevance of these molecules in CKD associated morbidity and mortality.

Keywords: biomarkers, chronic kidney disease, haemodialysis, mechanisms, proteomics, uraemic solutes

INTRODUCTION

Chronic kidney disease (CKD) is characterized by the 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. haemodialysis (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 (uraemic retention solutes) and drugs that are normally metabolized or excreted by the kidney. Such uraemic 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 progress in proteomics technologies and software solutions have enabled assessment of 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.

MATERIALS 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 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 haemodialysis underwent either haemodialysis or online-haemodiafiltration for 4 h three times 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 according to standardized procedures (http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures) prior to dialysis, before anticoagulant administration. One patient on home haemodialysis 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. Estimated glomerular filtration rate (eGFR) was calculated based on serum creatinine using the CKD epidemiology collaboration (CKD-EPI) creatinine equation. Proteinuria was determined using urine dipstick + or higher and/or with ≥0.25 g protein/g creatinine.

Sample preparation for proteomic analysis

Ten microlitres of thawed plasma sample was diluted with 90 µL 0.1% sodium dodecyl sulphate, 20 mM dithiothreitol and 0.1 M TrisHCl (pH = 7.6). The sample was sonicated at room temperature for 30 min, 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, 50 mM), applied to NAP-5 column, and eluted with 1 mL of 50 mM ammonium bicarbonate buffer solution.

Twenty micrograms 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 min rising to 25% B after 360 min then on to 65% B after 480 min.

Proteomics data processing

Peptide analysis was performed using SEQUEST against the Human Uniprot Database as described in [19].

The relative quantitative analysis was performed based on the peptide area values. Before analysis, ppm-normalization of peptide areas was conducted.

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

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 with 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-E12962 h)] and histidine-rich glycoprotein [Cusabio (CSB-E13159 h)] 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 haemodialysis for at least 1 year (CKD5/HD group) (Table 1). The workflow and design of the study is given in Figure 1. LC–MS/MS analysis enabled identification of 9017 peptides (Supplementary data, Table S1), representing a total of 2054 unique proteins quantified using a label-free approach (Supplementary data, Table S2). To assess the validity of the quantification strategy, the reported concentration of 20 high abundant plasma proteins [21–23] (Supplementary data, Table S3) was compared with their respective relative abundance in the LC–MS/MS experiment (normalized-ppm mass-peak area, arbitrary units). Significant correlation between existing data and LC-MS/MS quantification was observed (CKD2-3 Spearman $r = 0.8480$, $P < 0.0001$, CKD5/HD Spearman $r = 0.7889$, $P < 0.0001$, Figure 2A). 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 data sets 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 with CKD2-3 (Supplementary data, Table S2). 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 sub-cohort of seven CKD2-3 patients and seven CKD5/HD patients (Table 1). Although this sub-cohort comparison is of lower statistical power, the initial results could be generally confirmed (Table 2).

The LC–MS/MS data obtained enabled the confirmation of increased abundance of seven well-characterized uraemic toxins [11, 12] including beta-2-microglobulin, prostaglandin-H2 D-isomerase and cystatin-C (Table 3 and Supplementary data, Table S4). Their plasma accumulation was significantly correlated to renal function decline (Table 3). Two other uraemic toxins, immunoglobulins kappa and lambda, were detected but showed unmodified abundance (Supplementary data, Table S4).

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 signalling and complement and coagulation systems (Figures 3–4 and Supplementary data, Table S5). Other pathways included platelet degranulation, calcium ion-dependent exocytosis, response to selenium ion, liver X receptor (LXR)/retinoid R receptor (RXR) activation, atherosclerosis signalling and production of nitric oxide (NO) and reactive oxygen species (ROS) (Supplementary data, Table S5).

Of the 33 proteins associated with the complement system, 24 were detected in the plasma using LC–MS/MS. Among those, five were significantly increased in abundance in the plasma of CKD5/HD patients compared with CKD2-3 (Figure 3A), suggesting activation of the complement system in advanced CKD. Twenty-three out of 35 proteins associated with the coagulation system were detected in the plasma using LC–MS/MS (data not shown), and levels of five were significantly modified, three with lower and two with higher abundance in CKD5/HD compared with CKD2-3 (Figure 3B). From a total of 169 proteins identified to be associated with acute phase response signalling 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): nine proteins known to be increased in the plasma during the acute phase response were found to be more abundant, and four known to be decreased were found to be less abundant in CKD5/HD compared with 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 with CKD2-3 (Figure 4, Bold and Table 1), confirming activation of the acute phase response in CKD. To avoid introducing bias as a result of significantly upregulated CRP in the CKD5/HD group, the same analysis was performed after exclusion of six patients of the CKD5/HD group with highest CRP values. The CRP levels of this sub-cohort were 2.71 ± 2.71 mg/L for CKD2-3 and 3.95 ± 2.14 mg/L for CKD5/HD. Analysis of the proteome of this sub-cohort using IPA yielded similar results to those obtained when analysing the full cohort. We found that high or low CRP did not influence the results, as the top pathways were similar in both cases. In particular, acute phase response, coagulation system and complement system were among the top six pathways.

Table 1. Patient selection

	Discovery cohort (complete)			Discovery sub cohort (matched)			Validation cohort					
	CKD2-3	CKD5/HD	P-value	CKD2-3	CKD5/HD	P-value	Controls	CKD3b	CKD4	CKD5 no HD	CKD5/HD	P-value
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	<10	<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	3.37 ± 2.50	3.37 ± 1.30	4.12 ± 4.75	5.52 ± 4.44	n.s.
Aetiologies												
Diabetes	4	7	n.s.	3	4	n.s.	—	2	1	1	2	n.s. (between all
Vascular	4	3		4	2		—	0	3	1	2	groups)
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.
Anti-aggregant	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.

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.

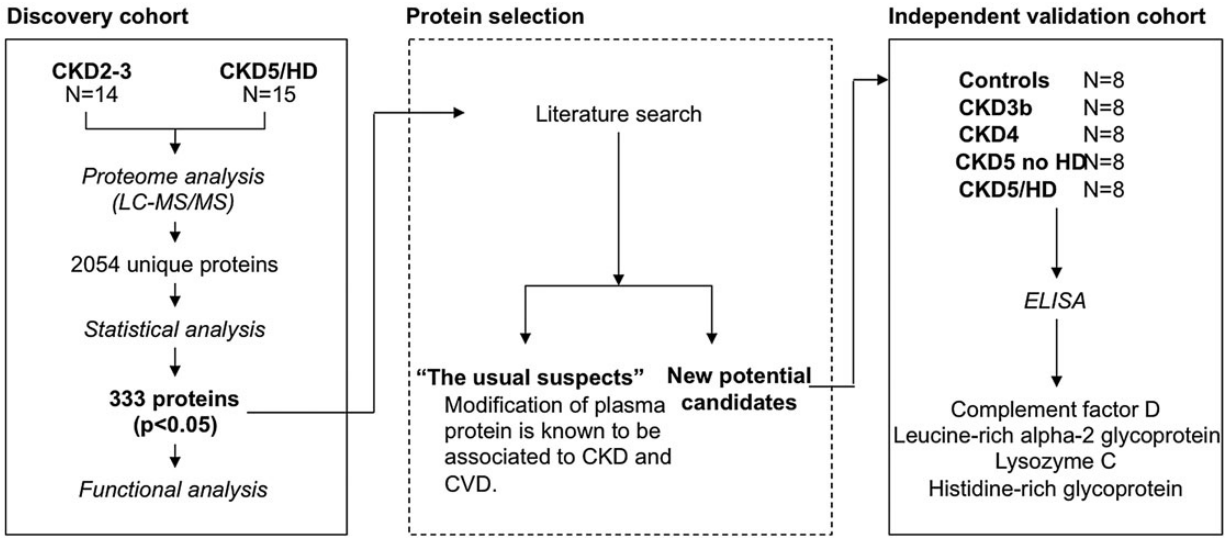

FIGURE 1: Overview of the study design.

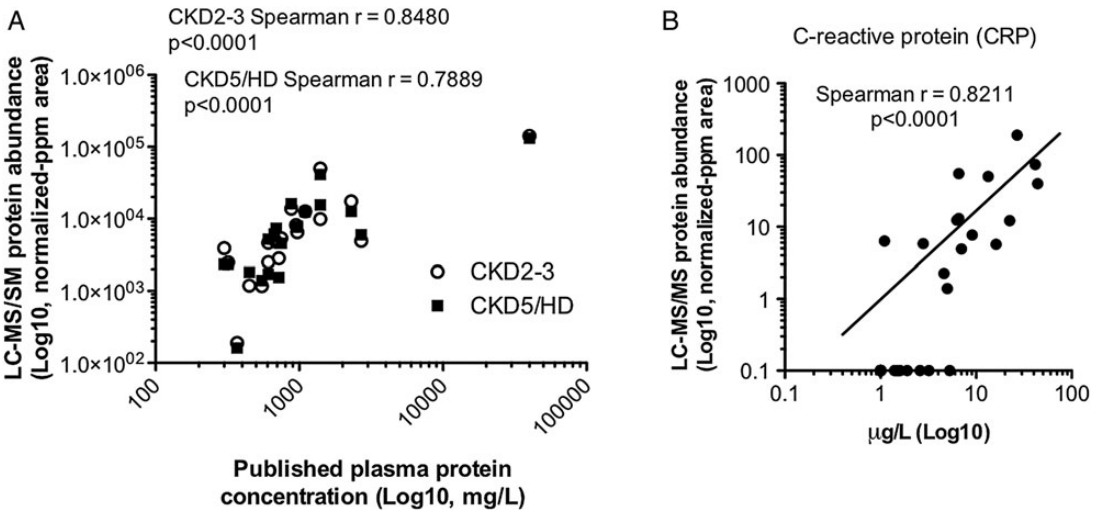

FIGURE 2: Correlation of quantification of plasma proteins identified using LC–MS/MS with their plasma concentrations. (**A**) Correlation of the published concentrations of the 20 most abundant plasma proteins as measured in the standard plasma using immune assays to their relative LC–MS/MS abundance 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.

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 with controls. We further showed that their abundance progressively increased during CKD progression (Figure 5A–C). We also demonstrated that protein levels were increased in the CKD5/HD group compared with CKD5 not on dialysis (Figure 5E–G). We could not validate the observed LC–MS/MS change for histidine-rich glycoprotein (Figure 5D and H). 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 data, 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 with all-cause mortality in CKD patients during a follow-up period of 3.1 ± 0.1 years post sampling

Table 2. Plasma proteome changes in CKD2-3 versus CKD5/HD patients

			Discovery cohort (complete)				Discovery sub-cohort (matched)		
Symbol	Name	Molecular weight (kDa)	Fold change	Direction	Unadjusted P-value	Adjusted P-value	Fold change	Direction	Unadjusted P-value
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	Leucocyte 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	Haemoglobin 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 intermediate peptidase	77	0.01	Down	0.0001	0.0105	0.01	Down	0.0421

List of 39 modified proteins identified with LC–MS/MS in the complete discovery cohort (adjusted P-value <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.

Table 3. Plasma accumulation of uraemic toxins

Uraemic toxin	Uniprot ID	MW (kDa)	CKD5/HD versus CKD2-3	Correlation to eGFR	
			Regulation	Rho	P-value
Alpha-1-acid glycoprotein 1	A1AG1_HUMAN	43	Up	−0.4621	0.0058
Protein AMBP [Cleaved into: Alpha-1-microglobulin	AMBP_HUMAN	33	Up	−0.8473	<0.0001
Prostaglandin-H2 D-isomerase (β-Trace protein)	PTGDS_HUMAN	26	Up	−0.8874	<0.0001
Beta-2-microglobulin	B2MG_HUMAN	12	Up	−0.8459	<0.0001
Complement factor D	CFAD_HUMAN	24	Up	−0.8245	<0.0001
Cystatin-C	CYTC_HUMAN	13	Up	−0.5665	0.0007
Retinol-binding protein 4	RET4_HUMAN	21	Up	−0.6488	<0.0001

List of seven modified uraemic toxins identified with LC–MS/MS in the complete discovery cohort and their correlation to eGFR.

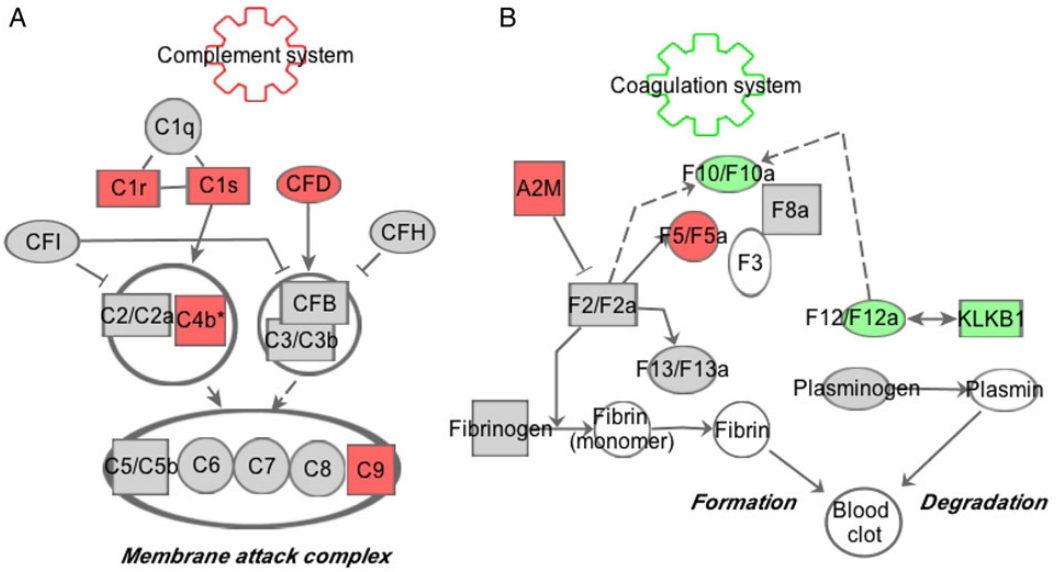

FIGURE 3: Alteration of the complement and coagulation systems. Simplified view of the complement (**A**) and coagulation (**B**) systems using Ingenuity pathway designer. Grey: not modified; Green: decreased abundance; Red: increased abundance; White: not detected with LC–MS/MS; Oval shape: pathway-specific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

(Figure 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. No significant association with mortality was detectable for complement factor D, lysozyme C and histidine-rich glycoprotein. In contrast, high abundance of leucine-rich alpha-2 glycoprotein was significantly associated with mortality in patients with CKD5 on haemodialysis compared with patients with low leucine-rich alpha-2 glycoprotein abundance (Figure 6C). The 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, not significant) nor by time on dialysis (2 ± 1.26 and 2 ± 1.15 years in low versus high, not significant).

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 improve our understanding of the pathophysiology of CKD. To address this question, 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).

Uraemic toxins are solutes normally excreted by the kidneys, which accumulate and negatively impact biological functions when renal function declines. Reviews from the European Uremic Toxin Work Group listed and classified 39 uraemic retention proteins and their normal and uraemic 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 uraemic solutes, including beta-2-microglobulin, prostaglandin-H2 D-isomerase and cystatin-C out of the 333 proteins that were significantly different between the two groups and showed that their plasma levels were correlated to renal function decline. Two additional toxins, immunoglobulins kappa and lambda, were detected using LC–MS/MS but were not accumulating in the plasma of CKD5/HD patients. We could not detect other known uraemic toxins most likely due to their very low molecular weight (e.g. proenkephalin-A) and/or their very low abundance (e.g.

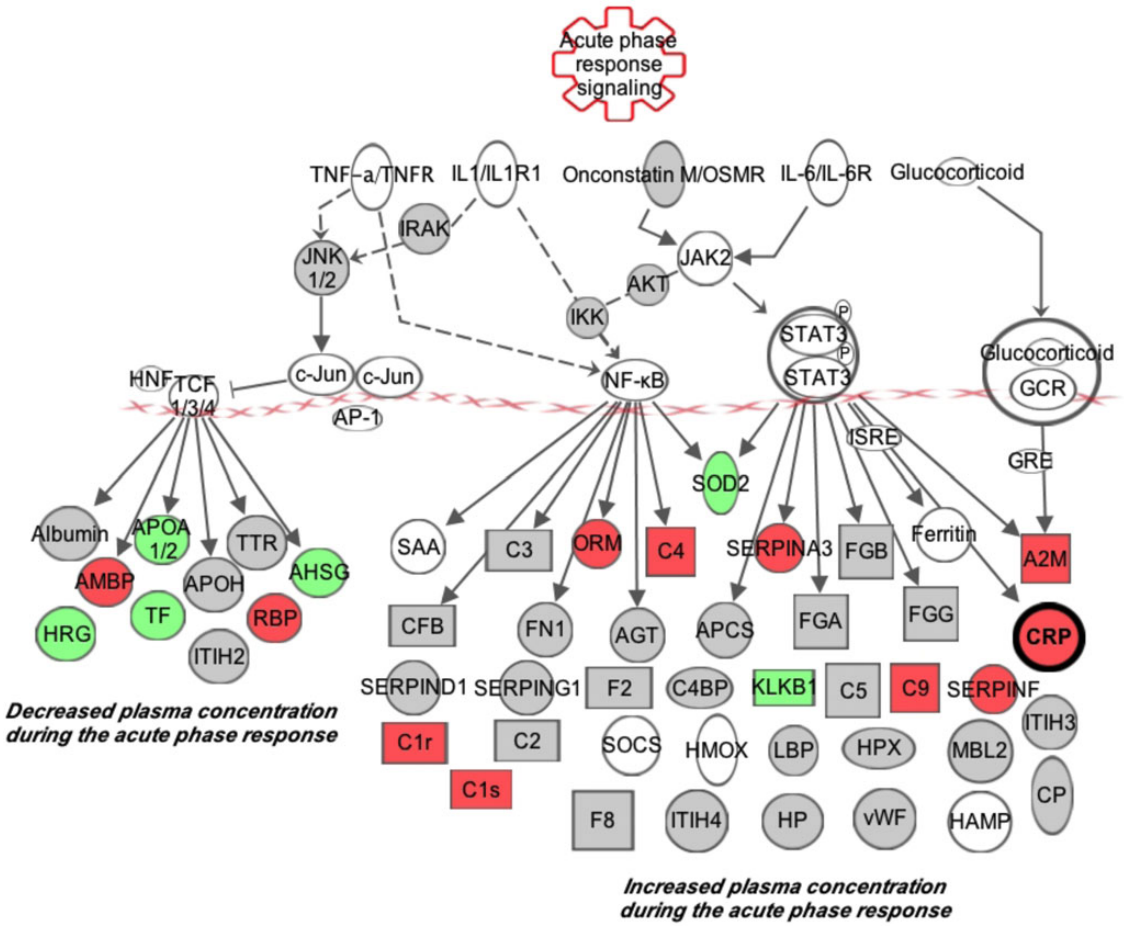

FIGURE 4: Alteration of the acute-phase response pathway. Simplified view of the acute-phase response signalling pathway using Ingenuity pathway designer. Grey: not modified; Green: decreased abundance; Red: increased abundance; White: not detected with LC–MS/MS; Oval shape: pathway-specific proteins;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

fibroblast growth factor 23) and the masking effect of highly abundant plasma proteins such as albumin (Supplementary data, Table S4).

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 with 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 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.

In our study, plasma accumulation of lysozyme C and leucine-rich alpha-2-glycoprotein was significantly correlated with renal function decline (Supplementary data, Figure), suggesting that the increased plasma levels of the molecules reflect at least in part alteration of glomerular filtration. Circulating lysozyme C, a protein released from leucocytes 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 poorly described function. It has been linked to different types 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 increased in the urine of patients with IgA nephropathy and CKD [37, 38]. Although we do not know whether plasma accumulation of lysozyme C and leucine-rich alpha-2-glycoprotein can be responsible for the development of complications in patients with advanced CKD, it is interesting to note that the 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 marker 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 insight, we

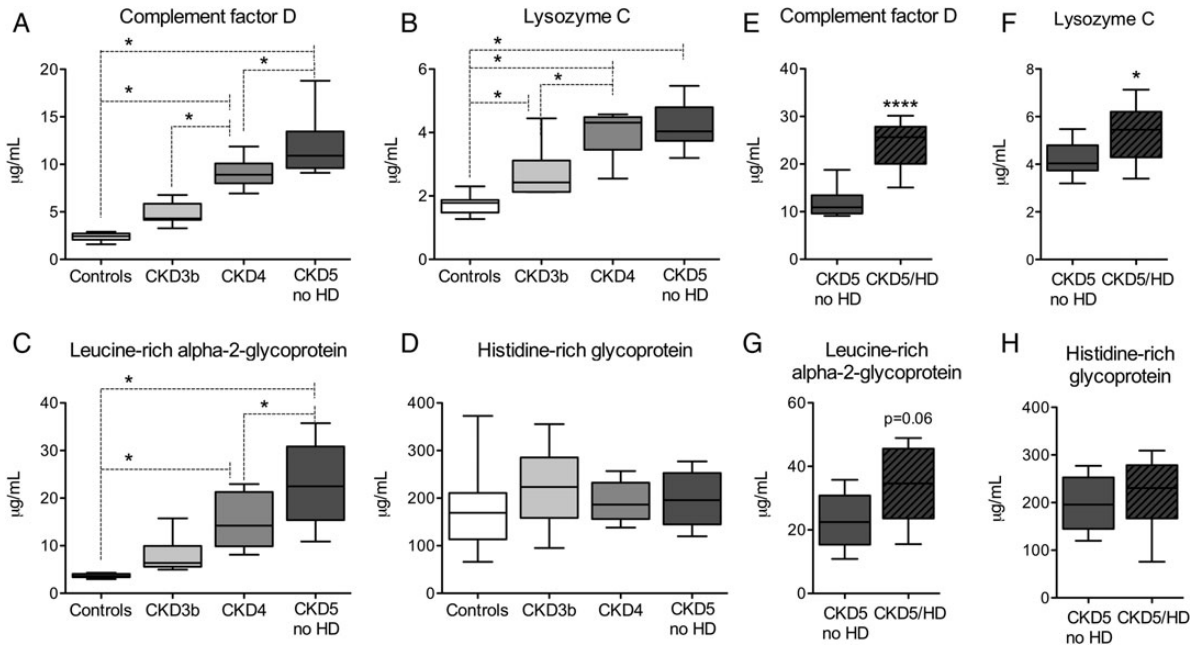

FIGURE 5: Validation of plasma protein modifications during CKD progression and HD. Plasma changes of complement factor D (**A** and **E**), lysozyme C (**B** and **F**), leucine-rich alpha-2-glycoprotein (**C** and **G**) and histidine-rich glycoprotein (**D** and **H**) identified with high confidence 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.

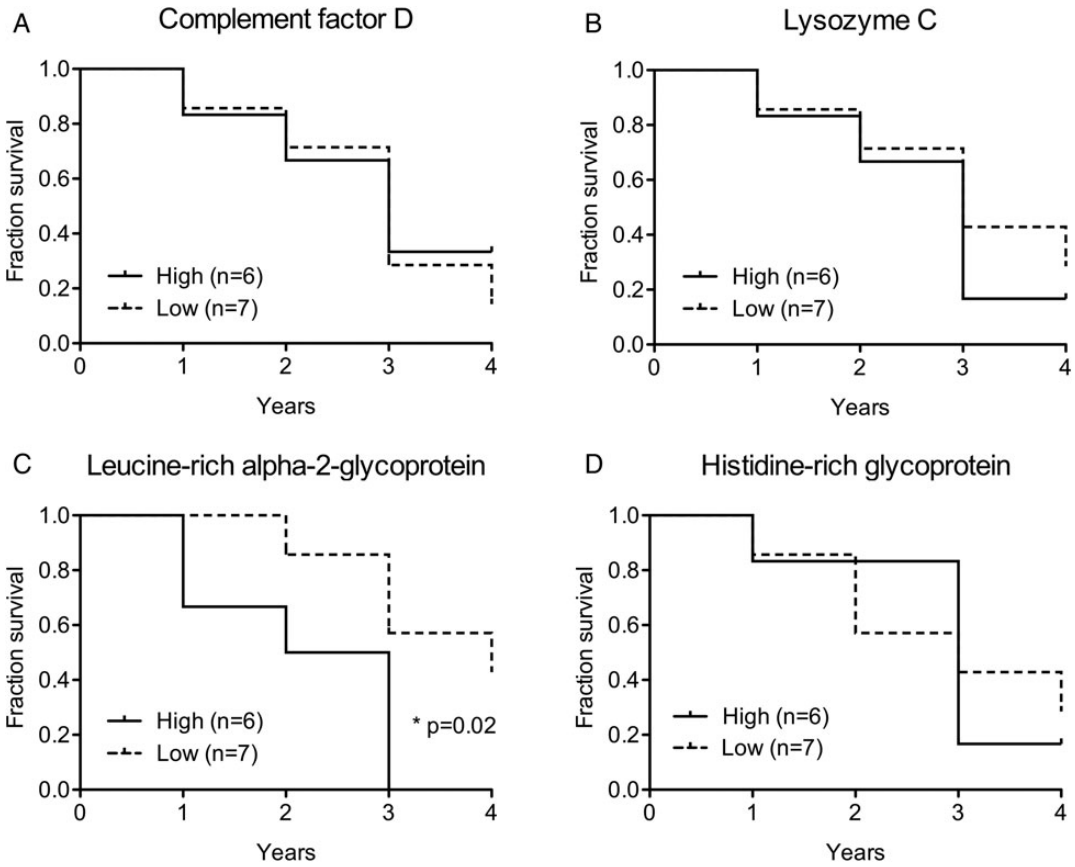

FIGURE 6: Association of plasma protein levels with mortality in CKD5/HD patients. Survival analysis was performed on CKD5/HD patients from the discovery cohort stratified based on high (solid line) versus low (dashed line) LC–MS/MS abundance of complement factor D (High $n = 6$; Low $n = 7$) (**A**), 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**).

assessed the cellular activities to which these proteins are related to and observed that many of them participate in acute phase response and complement and coagulation system activation (Figures 3–4, Supplementary data, Table S5). Systemic inflammation 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, indicating acute inflammatory response in uraemic conditions (Figure 4 and Supplementary data, Table S5). 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 with 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]. 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 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 appear indicated.

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 data, Table S5), were observed. Changes in haemostasis are well described in CKD [41] due to uraemic toxins, interaction with dialyser membranes and the repeated use of anticoagulants all together contributing to the risk of cardiovascular and thrombotic complications in patients undergoing HD [41]. Lower levels of F10 and KLKB1, together with increased levels of tissue factor pathway inhibitor (TFPI), a direct inhibitor of F10, have been described in patients with CKD and HD, a finding in accordance with our observed decrease of F10 and KLKB1 [45–48]. Moreover, the plasma level of F5 was found to be significantly increased in patients on HD, a finding again in accordance with our observed increased level of F5 in the plasma of CKD5/HD patients [49]. Regarding F12, the findings are more controversial. While F12 activity was decreased in patients with IgA nephropathy [48], F12 levels were found either not modified or increased in patients with ESRD and HD [46, 50, 51]. Further studies are thus required to investigate in detail the modifications of coagulation factors during CKD and HD.

One of the main limitations of this study is that observed molecular changes have not been related to clinical endpoints such as haemorrhages, 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 haemodialysis compared with patients with low leucine-rich alpha-2 glycoprotein abundance (Figure 6C). Indeed, this study was designed as a discovery study to first identify plasma proteins significantly changed in CKD. Other compounds and new hypotheses should be validated in other cohorts with appropriate statistical power to specifically address their potential association with CKD-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. Some of the significant changes were verified in an independent cohort, further supporting the validity of the proteomics data presented. 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 in CKD-associated morbidity and mortality.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

ACKNOWLEDGEMENTS

This study was funded in part by 'Clinical and system –omics for the identification of the Molecular Determinants of established Chronic Kidney Disease' (iMODE-CKD, PEOPLE-ITN-GA-2013-608332), 'Systems Biology to Identify Molecular Targets for Vascular Disease Treatment' (SysVasc, HEALTH-2013 603288), Urosysteomics (Urosysteomics ETB-2006-016) and 'Systems biology towards novel chronic kidney disease diagnosis and treatment' (SysKID HEALTH-F2-2009-241544). G.G., J.J, J.P.S., A.A, H.M. and R.V. are members of the European Uraemic Toxin Working Group of the European Society of Artificial Organs (ESAO) endorsed by the European Renal Association—European Dialysis Transplantation Association.

CONFLICT OF INTEREST STATEMENT

H.M. is the founder and co-owner of Mosaiques Diagnostics. The results presented in this paper have not been published previously in whole or part, except in abstract format.

REFERENCES

1. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 2002; 39: S1–S266
2. Coresh J, Selvin E, Stevens LA *et al.* Prevalence of chronic kidney disease in the United States. JAMA 2007; 298: 2038–2047
3. Dalrymple LS, Katz R, Kestenbaum B *et al.* Chronic kidney disease and the risk of end-stage renal disease versus death. J Gen Intern Med 2011; 26: 379–385
4. Moody WE, Edwards NC, Chue CD *et al.* Arterial disease in chronic kidney disease. Heart 2013; 99: 365–372
5. Matsushita K, van der Velde M, Astor BC *et al.* Association of estimated glomerular filtration rate and albuminuria with all-cause and

cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet 2010; 375: 2073–2081
6. Vanholder R, Massy Z, Argiles A *et al.* Chronic kidney disease as cause of cardiovascular morbidity and mortality. Nephrol Dial Transplant 2005; 20: 1048–1056
7. Bansal S, Siddarth M, Chawla D *et al.* Advanced glycation end products enhance reactive oxygen and nitrogen species generation in neutrophils in vitro. Mol Cell Biochem 2012; 361: 289–296
8. Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 2004; 63: 582–592
9. Nguyen-Khoa T, Massy ZA, De Bandt JP *et al.* Oxidative stress and haemodialysis: role of inflammation and duration of dialysis treatment. Nephrol Dial Transplant 2001; 16: 335–340
10. Afsar B, Turkmen K, Covic A *et al.* An update on coronary artery disease and chronic kidney disease. Int J Nephrol 2014; 2014: 767424
11. Duranton F, Cohen G, De Smet R *et al.* Normal and pathologic concentrations of uremic toxins. J Am Soc Nephrol 2012; 23: 1258–1270
12. Vanholder R, De Smet R, Glorieux G *et al.* Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int 2003; 63: 1934–1943
13. Liabeuf S, Lenglet A, Desjardins L *et al.* Plasma beta-2 microglobulin is associated with cardiovascular disease in uremic patients. Kidney Int 2012; 82: 1297–1303
14. Lin Z, Zhou Z, Liu Y *et al.* Circulating FGF21 levels are progressively increased from the early to end stages of chronic kidney diseases and are associated with renal function in Chinese. PLoS One 2011; 6: e18398
15. Malickova K, Brodska H, Lachmanova J *et al.* Plasma calprotectin in chronically dialyzed end-stage renal disease patients. Inflamm Res 2010; 59: 299–305
16. Sabbisetti VS, Waikar SS, Antoine DJ *et al.* Blood kidney injury molecule-1 is a biomarker of acute and chronic kidney injury and predicts progression to ESRD in type I diabetes. J Am Soc Nephrol 2014; 25: 2177–2186
17. Celap I, Simundic AM, Nikolac N *et al.* Visfatin is not associated with inflammatory markers in patients on hemodialysis. Clin Lab 2013; 59: 1253–1259
18. Yilmaz MI, Siriopol D, Saglam M *et al.* Plasma endocan levels associate with inflammation, vascular abnormalities, cardiovascular events, and survival in chronic kidney disease. Kidney Int 2014; 86: 1213–1220
19. Husi H, Sanchez-Nino MD, Delles C *et al.* A combinatorial approach of Proteomics and Systems Biology in unravelling the mechanisms of acute kidney injury (AKI): involvement of NMDA receptor GRIN1 in murine AKI. BMC Syst Biol 2013; 7: 110
20. Husi H, Van Agtmael T, Mullen W *et al.* Proteome-based systems biology analysis of the diabetic mouse aorta reveals major changes in fatty acid biosynthesis as potential hallmark in diabetes mellitus-associated vascular disease. Circ Cardiovasc Genet 2014; 7: 161–170
21. Haab BB, Geierstanger BH, Michailidis G *et al.* Immunoassay and antibody microarray analysis of the HUPO Plasma Proteome Project reference specimens: systematic variation between sample types and calibration of mass spectrometry data. Proteomics 2005; 5: 3278–3291
22. Hortin GL, Sviridov D, Anderson NL. High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. Clin Chem 2008; 54: 1608–1616
23. Polanski M, Anderson NL. A list of candidate cancer biomarkers for targeted proteomics. Biomark Insights 2007; 1: 1–48
24. Andersen RF, Palmfeldt J, Jespersen B *et al.* Plasma and urine proteomic profiles in childhood idiopathic nephrotic syndrome. Proteomics Clin Appl 2012; 6: 382–393
25. Majek P, Riedelova-Reicheltova Z, Suttnar J *et al.* Proteome changes in the plasma of myelodysplastic syndrome patients with refractory anemia with excess blasts subtype 2. Dis Markers 2014; 2014: 178709
26. Xu DD, Deng DF, Li X *et al.* Discovery and identification of serum potential biomarkers for pulmonary tuberculosis using iTRAQ-coupled two-dimensional LC-MS/MS. Proteomics 2014; 14: 322–331
27. Gotes J, Kasian K, Jacobs H *et al.* Lysozyme, a mediator of sepsis that deposits in the systemic vasculature and kidney as a possible mechanism of acute organ dysfunction. Shock 2014; 41: 256–265
28. Mink SN, Kasian K, Santos Martinez LE *et al.* Lysozyme, a mediator of sepsis that produces vasodilation by hydrogen peroxide signaling in an arterial preparation. Am J Physiol Heart Circ Physiol 2008; 294: H1724–H1735
29. Watson CJ, Ledwidge MT, Phelan D *et al.* Proteomic analysis of coronary sinus serum reveals leucine-rich alpha2-glycoprotein as a novel biomarker of ventricular dysfunction and heart failure. Circ Heart Fail 2011; 4: 188–197
30. Linden M, Lind SB, Mayrhofer C *et al.* Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer. Proteomics 2012; 12: 135–144
31. Wen SY, Zhang LN, Yang XM *et al.* LRG1 is an independent prognostic factor for endometrial carcinoma. Tumour Biol 2014; 35: 7125–7133
32. He X, Wang Y, Zhang W *et al.* Screening differential expression of serum proteins in AFP-negative HBV-related hepatocellular carcinoma using iTRAQ -MALDI-MS/MS. Neoplasma 2014; 61: 17–26
33. Kentsis A, Ahmed S, Kurek K *et al.* Detection and diagnostic value of urine leucine-rich alpha-2-glycoprotein in children with suspected acute appendicitis. Ann Emerg Med 2012; 60: 78–83 e71
34. Serada S, Fujimoto M, Terabe F *et al.* Serum leucine-rich alpha-2 glycoprotein is a disease activity biomarker in ulcerative colitis. Inflamm Bowel Dis 2012; 18: 2169–2179
35. Wang X, Abraham S, McKenzie JA *et al.* LRG1 promotes angiogenesis by modulating endothelial TGF-beta signalling. Nature 2013; 499: 306–311
36. Michel JB, Martin-Ventura JL, Nicoletti A *et al.* Pathology of human plaque vulnerability: mechanisms and consequences of intraplaque haemorrhages. Atherosclerosis 2014; 234: 311–319
37. Kalantari S, Rutishauser D, Samavat S *et al.* Urinary prognostic biomarkers and classification of IgA nephropathy by high resolution mass spectrometry coupled with liquid chromatography. PLoS One 2013; 8: e80830
38. Vivekanandan-Giri A, Slocum JL, Buller CL *et al.* Urine glycoprotein profile reveals novel markers for chronic kidney disease. Int J Proteomics 2011; 2011: 214715
39. Abdul-Salam VB, Ramrakha P, Krishnan U *et al.* Identification and assessment of plasma lysozyme as a putative biomarker of atherosclerosis. Arterioscler Thromb Vasc Biol 2010; 30: 1027–1033
40. Romao JE, Jr, Haiashi AR, Elias RM *et al.* Positive acute-phase inflammatory markers in different stages of chronic kidney disease. Am J Nephrol 2006; 26: 59–66
41. Schoorl M, Grooteman MP, Bartels PC *et al.* Aspects of platelet disturbances in haemodialysis patients. Clin Kidney J 2013; 6: 266–271
42. Volanakis JE, Barnum SR, Giddens M *et al.* Renal filtration and catabolism of complement protein D. N Engl J Med 1985; 312: 395–399
43. Beazley KE, Reckard S, Nurminsky D *et al.* Two sides of MGP null arterial disease: chondrogenic lesions dependent on transglutaminase 2 and elastin fragmentation associated with induction of adipsin. J Biol Chem 2013; 288: 31400–31408
44. Zhu L, Wigle D, Hinek A *et al.* The endogenous vascular elastase that governs development and progression of monocrotaline-induced pulmonary hypertension in rats is a novel enzyme related to the serine proteinase adipsin. J Clin Invest 1994; 94: 1163–1171
45. Adams MJ, Irish AB, Watts GF *et al.* Hypercoagulability in chronic kidney disease is associated with coagulation activation but not endothelial function. Thromb Res 2008; 123: 374–380
46. Matsuo T, Koide M, Kario K *et al.* Extrinsic coagulation factors and tissue factor pathway inhibitor in end-stage chronic renal failure. Haemostasis 1997; 27: 163–167
47. Sioulis A, Malindretos P, Makedou A *et al.* Coagulation factors as biological risk markers of endothelial dysfunction. Association with the thrombotic episodes of chronic hemodialysis patients. Hippokratia 2009; 13: 237–241
48. Tan CC, Lee GS, Lee EJ *et al.* Plasma activity of contact coagulation factors in patients with IgA nephritis. Ann Acad Med Singapore 1996; 25: 218–221
49. Deguchi K, Izumi K, Noguchi M *et al.* Coagulation and fibrinolysis in patients with chronic renal failure on maintenance hemodialysis. Nihon Ketsueki Gakkai Zasshi 1989; 52: 140–146
50. Oda H, Ohno M, Ohashi H. Coagulation and fibrinolysis factors in dialysis patients with and without ischemic heart disease. Adv Perit Dial 2000; 16: 152–155
51. Papageorgiou PC, Chan CT, Yeo EL *et al.* Coagulation Factor XIIa-kinin-mediated contribution to hypertension of chronic kidney disease. J Hypertens 2014; 32: 1523–1533; discussion 1533

Received for publication: 23.1.2015; Accepted in revised form: 21.5.2015