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

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The rationale of the present study was to apply untargeted high-resolution plasma proteome analysis to investigate molecular changes associated with end stage renal disease (ESRD). Our aim was to identify proteins with altered plasma levels and estimate the potential functional consequences of such changes using a systems biology approach.

1 Plasma proteome analysis in patients with CKD

We studied 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).

LC-MS/MS analysis of the 29 plasma samples (sampled prior to HD in CKD5/HD patients) enabled identification of 9017 peptides, representing a total of 2054 unique proteins quantified using a label-free approach.

In order to validate our proteome-based approach we determined the correlation between plasma C-reactive protein (CRP) measured using routine clinical laboratory analysis and proteome-based analysis (Figure 1). A significant correlation between quantitative measurement of CRP and semi-quantitative LC-MS/MS abundance in the discovery cohort was observed.

Table 1. Patient selection

	CKD2-3	CKD5/HD	pValue
N	14	15	-
Male/Female	6/8	12/3	0.039
eGFR (mL/min/1.73m²)	63.78 ±14.42	9.13 ± 3.20	<0.0001
Age (years)	57.16 ±11.63	70.33 ±8.49	0.004
Time on dialysis (year)	-	2.58 ±2.57	-
BMI (kg/m²)	29.39 ±6.79	25.81 ±4.78	n.s.
Systolic blood pressure (mmHg)	134±15	150±19	0.032
Diastolic blood pressure (mmHg)	71±8	77 ±9	n.s.
C-Reactive Protein (mg/L)	2.71 ±2.71	13.35 ±14.26	0.003
Etiologies			
Diabetes	4	7	n.s.
Vascular	4	3	
Glomerular	1	2	
ADPKD	0	0	
Other/Unknown	5	3	
Medications (%)			
Vitamin K anta	7	7	n.s.
Antiaggregant	0	53	0.0022
ACEi/ARB	36	33	n.s.
Statin	21	33	n.s.
Cortisone	0	0	n.s.

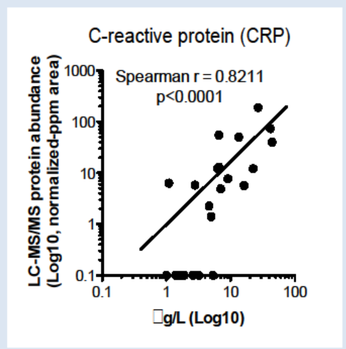

Figure 1. Correlation of the absolute concentration of C-reactive protein (CRP) to its relative LC-MS/MS abundance in the plasma of the CKD2-3 or CKD5/HD patients

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

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. Upon correction for multiple testing, 39 of the 333 remained significant (Table 2). **Several of these proteins were previously described as being modified in ESRD (e.g. beta-2-microglobulin, prostaglandin-H2 D-isomerase).**

Table 2. Plasma proteome changes in CKD patients. ●: proteins validated using ELISA (section 3)

Symbol	Name	Molecular weight (kDa)	Fold change	Direction	Unadjusted pValue	Adjusted pValue
IGFBP6	Insulin-like growth factor-binding protein 6	23	n.d. in CKD2-3	Up	1,00E-04	1,28E-02
● LYZ	Lysozyme C	15	n.d. in CKD2-3	Up	2,00E-04	1,87E-02
B2M	Beta-2-microglobulin	12	99,03	Up	4,92E-05	1,05E-02
● CFD	Complement factor D	24	97,8	Up	1,62E-05	4,40E-03
PTGDS	Prostaglandin-H2 D-isomerase	19	32,36	Up	3,33E-04	2,45E-02
PRPF3	U4/U6 small nuclear ribonucleoprotein Prp3	78	28,16	Up	1,31E-04	1,50E-02
WDFY4	WD repeat- and FYVE domain-containing protein 4	354	7,82	Up	2,38E-04	2,04E-02
RUSC2	Iporin	161	6,02	Up	1,19E-06	1,22E-03
C1S	Complement C1s subcomponent	47 & 28	4,54	Up	9,81E-05	1,28E-02
SSX2IP	Afadin- and alpha-actinin-binding protein	71	4,53	Up	6,13E-05	1,14E-02
LENG8	Leukocyte receptor cluster member 8	86	4,41	Up	1,71E-05	4,40E-03
AMBP	Protein AMBP	21 & 16 & 7	3,98	Up	7,55E-06	3,88E-03
CCDC147	Coiled-coil domain-containing protein 147	103	3,57	Up	1,84E-04	1,80E-02
SETD2	Histone-lysine N-methyltransferase SETD2	288	3,37	Up	9,48E-04	4,99E-02
NR0B1	Nuclear receptor subfamily 0 group B member 1	52	3,15	Up	9,19E-05	1,28E-02
C1R	Complement C1r subcomponent	51 & 27	3	Up	7,17E-05	1,23E-02
HBB	Hemoglobin subunit beta	16	2,85	Up	6,23E-04	4,03E-02
CNNM4	Metal transporter CNNM4	87	2,55	Up	2,37E-04	2,04E-02
SLC9A5	Sodium/hydrogen exchanger 5	99	2,23	Up	1,54E-04	1,67E-02
TFAM	Transcription factor A, mitochondrial	24	2,15	Up	7,06E-04	4,27E-02
ITPR1	Inositol 1,4,5-trisphosphate receptor type 1	314	2,01	Up	5,56E-04	3,94E-02
SERPINF1	Pigment epithelium-derived factor	44	1,92	Up	2,89E-04	2,28E-02
IGHA1	Ig alpha-1 chain C region	38	1,88	Up	6,09E-04	4,03E-02
CSPP1	Centrosome and spindle pole-associated protein 1	146	0,64	Down	7,82E-04	4,59E-02
LRRIQ3	Leucine-rich repeat and IQ domain-containing protein 3	74	0,6	Down	2,55E-04	2,10E-02
FNBP1	Formin-binding protein 1	71	0,54	Down	1,11E-04	1,34E-02
RLTPR	Leucine-rich repeat-containing protein 16C	155	0,5	Down	3,18E-04	2,42E-02
● HRG	Histidine-rich glycoprotein	58	0,47	Down	6,27E-04	4,03E-02
F12	Coagulation factor XII	40 & 26	0,46	Down	8,51E-04	4,72E-02
ARHGAP25	Rho GTPase-activating protein 25	73	0,42	Down	8,47E-04	4,72E-02
SNIP1	Smad nuclear-interacting protein 1	46	0,37	Down	1,75E-04	1,79E-02
ZNF415	Zinc finger protein 415	69	0,3	Down	6,96E-04	4,27E-02
CALR3	Calreticulin-3	43	0,29	Down	9,43E-04	4,99E-02
ZFAND4	AN1-type zinc finger protein 4	80	0,23	Down	1,99E-06	1,37E-03
NOS2	Nitric oxide synthase, inducible	131	0,16	Down	1,63E-05	4,40E-03
GPX3	Glutathione peroxidase 3	23	0,15	Down	8,03E-07	1,22E-03
PDE4B	cAMP-specific 3,5-cyclic phosphodiesterase 4B	83	0,15	Down	8,64E-05	1,28E-02
SBF1	Myotubularin-related protein 5	208	0,05	Down	1,08E-05	4,40E-03
MIPEP	Mitochondrial intermediate peptidase	77	0,01	Down	5,10E-05	1,05E-02

Using a systems biology approach, the functional impact of the 333 proteins suggests modifications of pathological processes playing a role in the **complications of ESRD such as acute phase response, complement and coagulation systems** (Figure 2).

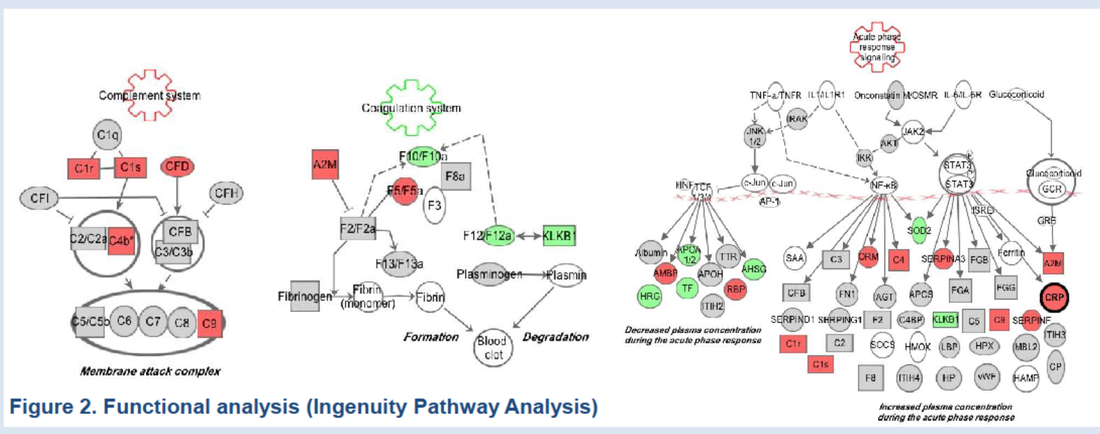

Figure 2. Functional analysis (Ingenuity Pathway Analysis)

3 Validation of newly identified plasma proteins using ELISA

We aimed to validate some of these changes 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.

We selected **lysozyme C, leucine-rich alpha-2 glycoprotein** and **histidine-rich glycoprotein** (Figure 3) since they have been related to vascular dysfunction and heart failure and were not yet described in the context of CKD. Moreover, we selected **complement factor D** (Figure 3), since our data indicated that the complement system was activated in CKD (Figure 2).

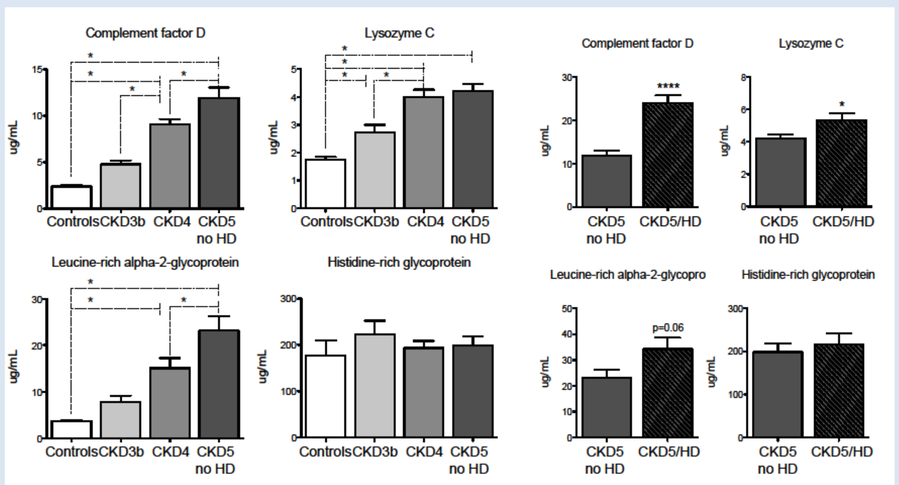

Figure 3. Validation of plasma protein modifications during CKD and HD

The abundance of complement factor D, lysozyme C and leucine-rich alpha-2 glycoprotein **progressively increased during CKD progression**. Their abundance were inversely correlated with eGFR (not shown). We could not validate LC-MS/MS change for histidine-rich glycoprotein.

4 Association 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. 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.

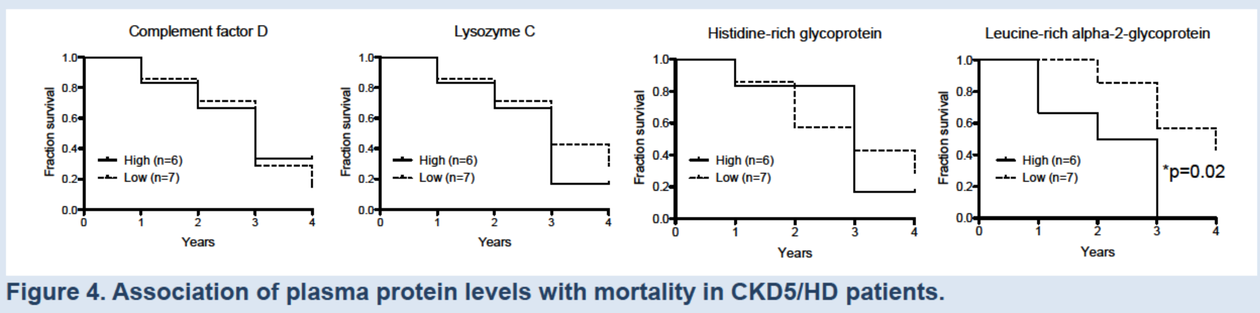

Figure 4. Association of plasma protein levels with mortality in CKD5/HD patients.

High abundance of leucine-rich alpha-2 glycoprotein was significantly associated with mortality compared to patients with low leucine-rich alpha-2 glycoprotein abundance (Figure 4). 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).

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/ESRD-associated morbidity and mortality.

52 ERA 279--FP

J2) Chronic Kidney Disease. Pathophysiology, progression & risk factors.

Klein Julie

DOI: 10.3252/pso.eu.52era.2015

ePosters supported by F. Hoffmann- La Roche Ltd.

52nd ERA-EDTA CONGRESS London, UNITED KINGDOM MAY 28 '31

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