Aus der Klinik für Nephrologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

The role of urinary peptide markers in diagnosis and prognosis of severe renal diseases

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Martin Pejchinovski

aus Skopje, Mazedonien

Datum der Promotion: 09.12.2016

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List of abbreviations

ADPKD- autosomal polycystic kidney disease; **ESRD**- end stage renal disease; **htTKV**-high- adjusted total kidney volume; **MS**- mass spectrometry; **CE-MS**- capillary electrophoresis couple to mass spectrometry; **SVM**- support vector machine; **AUC**-area under the ROC curve; **ROC**-receiver operating characteristics; **AKI**-acute kidney injury; **CKD**-chronic kidney disease; **RIFLE**- risk/failure/loss of kidney function/end stage renal disease; **MALDI**-matrix assisted laser desorption/ionization mass spectrometry; **LC-MS**- liquid chromatography couple to mass spectrometry; **HCD**-higher energy collision dissociation; **CID**- collision induced dissociation; **NOP**-naturally occurring peptide; **ITMS**- ion trap mass spectrometry; **FTMS**- fourier transform mass spectrometry; **FDR**- false discovery rate; **Xcorr**- cross-correlation.

Abstract (English)

Introduction: Early diagnosis and prognosis of kidney malfunction are of utmost interest for patient healthcare. Unfortunately, current clinical measurements and biochemical markers of the kidney function have provided the poor outcome of disease onset. Therefore, novel and more reliable biomarkers are needed to allow early risk stratification of the patients suffering from Autosomal polycystic kidney disease (ADPKD) and Acute kidney injury (AKI). With the usage of advances in mass spectrometry (MS)-based technology, screening of the urine proteome appears beneficial. Therefore, in this dissertation new and improved urinary peptide markers on different MS-based platforms were examined.

Methods: Urine samples from healthy, ADPKD and AKI patients were measured to analyse low molecular weight proteome and to establish a multidimensional peptide marker panel. For this purpose, capillary electrophoresis coupled to mass spectrometry (CE-MS), liquid chromatography coupled to mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization coupled to mass spectrometry (MALDI-MS) were applied.

Results: In the first part of the thesis 20 urinary peptides were identified by comparison of ADPKD patients exhibiting a relatively stable renal function with those progressing to end stage renal disease (ESRD) and multidimensional marker panel was established. Applied to several validation cohorts the panel achieved an area under the curve (AUC) between 0.83 and 0.92. Based on peptide sequence information *in silico* prediction of several proteases that may drive ADPKD progression was assessed.

In the second part, development of AKI classifier on MALDI MS-based platform was performed. This panel was generated by comparison of non-AKI and AKI patients, where 39 urinary peptide markers were selected. The performance of the peptide marker panel for early diagnosis of AKI was tested in the validation cohort and it achieved AUC 0.82.

The last part of the thesis was defined to investigate high energy collision dissociation (HCD) and collision-induced dissociation (CID) high resolution MS/MS who provided superior results in quantification and identification of naturally occurring peptides (NOPs) in urine. In addition, they demonstrated improvement of the correct peptide marker hits used in clinical biomarker discovery.

Conclusion: The limitations and increased non-effectiveness of quality management of patients in daily clinical practice, raise the possibility of proteomic analysis of urine to be utilized for routine health check-up providing earlier and more accurate identification of disease-related changes. The results of this thesis just demonstrated that mass spectrometry-based platforms could assist in better diagnostic and prognostic tests applicable for the clinical settings.

Abstract (German)

Einleitung: Eine frühzeitige Diagnose und Prognose von Nierenfunktionsstörungen ist für die Gesundheitsvor- und –fürsorge bei betroffenen Patienten von größtem Interesse. Die aktuellen klinischen Messungen und biochemischen Marker der Nierenfunktion sind dahingehend jedoch unzureichend. Daher sind neue und zuverlässigere Biomarker notwendig, um eine frühzeitige Risiko Stratifizierung z.B. von Patienten mit einer autosomal polyzystischen Nierenerkrankung (ADPKD) oder akutem Nierenversagen (AKI) zu ermöglichen. Durch die technologischen und analytischen Fortschritte in der Massenspektrometrie (MS) erschien eine Untersuchung des Urinproteoms vorteilhaft. Aus diesem Grund wurden in diesem Promotionsvorhaben neue und verbesserte Urin-Peptidmarker auf verschiedenen MS-basierten Plattformen analysiert.
Methoden: Die Urinproben von gesunden Personen sowie von ADPKD und AKI Patienten wurden massenspektrometrisch gemessen, um das niedrigmolekulare (≤ 20kDa) Proteom zu analysieren und multidimensionale Peptidmarkermuster zu etablieren. Hierzu wurden die direkt mit einer time-of-flight Massenspektrometrie gekoppelte Kapillarelektrophorese (CE-MS), die massenspektrometrie-gekoppelte Flüssigkeitschromatographie (LC-MS) und die matrix-unterstützte Laser-Desorption / Ionisation gekoppelte Massenspektrometrie (MALDI-MS) verwendet.
Ergebnisse: Im ersten Teil der Arbeit wurden 20 Urinpeptide durch den Vergleich von ADPKD Patienten mit einer relativ stabilen Nierenfunktion mit Patienten mit einer Nierenerkrankung im Endstadium (ESRD) identifiziert. Aus diesen wurde dann ein multidimensionales Markermuster erstellt. Dieses wurde auf mehrere Validierungskohorten angewendet und erreichte eine Fläche unter der Kurve (AUC) zwischen 0,83 und 0,92. Basierend auf Peptidsequenzinformationen erfolgte *in silico* die Vorhersage von mehreren Proteasen, die die Progression einer ADPKD vorantreiben könnten.

Im zweiten Teil der Arbeit wurde ein auf einer MALDI-MS-Plattform basierender AKI Klassifikator entwickelt. Das dazugehörige Markermuster wurde durch den Vergleich von nicht-AKI und AKI Patienten erzeugt, wobei 39 Urin-Peptidmarker identifiziert wurden. Die Leistung dieses Peptidmarkermusters für eine frühzeitige Diagnose von AKI wurde in der Validierungskohorte getestet und erreichte eine AUC von 0,82.

Im letzten Teil der Arbeit wurde eine hochauflösende MS/MS basierend auf Hochenergie-Kollisions Dissoziation (HCD) und stoßinduzierte Dissoziation (CID) verwendet, um hervorragende Ergebnisse bei der Quantifizierung und Identifizierung von natürlich vorkommenden Peptiden (NOP) im Urin zu erreichen. Darüber hinaus zeigten diese Analysen eine Verbesserung bei den Treffern für korrekte Peptidmarker in der klinischen Biomarker-Identifizierung.

Fazit: Die Möglichkeiten der der Proteom-Analyse in Urin im Rahmen einer Routinegesundheitsuntersuchung liegen in der Überwindung der begrenzten Effektivität und Wirksamkeit des momentanen Qualitätsmanagements z.B. bei ADPKD und AKI Patienten in der täglichen klinischen Praxis unter anderem durch die frühere und genauere Identifizierung von krankheitsbedingten Veränderungen. Die Ergebnisse dieses Promotionsvorhabens zeigen, dass die Analyse durch massenspektrometriebasierte Plattformen zu besseren diagnostischen und prognostischen Tests zur klinischen Patientenbeurteilung beitragen kann.

1. Introduction

According to the latest information provided by the European Society of Nephrologists, approximately 70 million Europeans have been reported with some loss of their kidney function and are exposed to a high risk for dialysis or renal transplantation (www.era-edta2015.org/press/1_150526_18.00_Press%20Release_CKD_Challenge.pdf). Among them are patients diagnosed with autosomal polycystic kidney disease (ADPKD) with an incidence between 1:400 and 1:1000 in the general population [1,2] and acute kidney injury (AKI) with a 2.5% of cases reported annually [3]. The both tubular diseases are characterized by a significant increase in serum creatinine levels typically followed by a rapid decline of glomerulus filtration rate (GFR) and progression to end stage renal disease (ESRD), where severe complication occurs and hamper a reliable clinical assessment. Therefore, this work was designed to investigate non-invasive diagnostic and prognostic urinary biomarker assays by different proteomic platforms that will help in better and more cost-effective medical care of the ill patients.

The ultimate goal of interdisciplinary care clinics is to provide well-timed prevention from the therapeutic intervention and/or keeping the long-term function of the kidneys. This can be done by early risk stratification of the patients being likely exposed to progress toward ESRD or renal failure. The symptoms of renal dysfunction often do not appear until subsequent kidney damage occur and their diagnostic or prognostic value remain scarce. Standard medical drugs like rigorous blood pressure control using renin-angiotensin-aldosterone (RAAS)-blockade, angiotensin-covering enzyme (ACE) and angiotensin receptor blockers (ARBs) have been shown to ease down the episodes of renal injury and delay disease progression in patients with renal tubular disorders [4-6]. In contrast, prolonged usage of these drugs could cause disease remission and a higher rate of side-effects [7,8]. Although they have been largely used as prevention therapy to slow down progressiveness in patients with moderate kidney function, their long term effects are missing. Therefore, novel and more reliable therapeutic targets are of utmost interest for improvement of diagnostic and prognostic accuracy in daily clinical practice.

Generally, the most common biomarkers used by the clinical centers for diagnostic and prognostic purposes are limited and suffer from inaccuracies. The reasons for such

insensitivities could be explained by the high variability of several associated factors common among individuals. The frequently used biomarkers for renal disease and progression to chronic kidney diseases (CKD) such as albuminuria/proteinuria and serum creatinine displayed a mild correlation with disease progression and demonstrated insufficient predictive power [9]. Existing functional parameters, estimated glomerular filtration rate (eGFR), widely used by the clinicians for monitoring of the kidney function, exhibit some inappropriate equation calculations and false evaluation of the clinical condition. In the same manner, measurements of total kidney volume adjusted for height (htTKV) mostly used as a prognostic marker for ADPKD requires specific imaging protocols not available to all clinics. In addition to this, rare disease indications and complex pathophysiological processes indicative of biological changes suggest utilization of biomarker panels as a necessary approach which might have a central role in transmitting the first information of disease activity, particularly when clinical endpoints are hard to predict. In such cases, biomarker panels could be far more informative, accurate and straightforward than the available clinical parameters. With the support of the latest proteomics platforms for qualitative and quantitative investigations of the adverse disease effects, translation of the scientific pathophysiological knowledge would be one step forward.

During past years, several proteomic analytical platforms became essential for the life science research and have sparked the great interest of clinical proteomics as a key component for improving patient healthcare. Capillary electrophoresis coupled to mass spectrometry (CE-MS), liquid chromatography coupled to mass spectrometry (LC-MS) and matrix-assist desorption/ionization time of flight (MALDI-TOF) mass spectrometry are especially important for measuring the diagnostic and prognostic proteomic/peptidomic signature associated with the development and progression of various diseases [10-12]. Human urine proteome displays the great potential to provide valuable immediate information about disease state present in the organism. Both methods are capable of analyzing low-molecular weight urinary proteins (up to 20kDa) and help medical representatives provide information in the context of clinical settings. Basically, they offer fast, sensitive, reproducibly, high–throughput and cost-effective assays that were used in this work to assess their role for diagnostic and prognostic purposes.

The first part of the thesis was focused on identification of novel peptide biomarkers in urine samples that predict the risk of reaching ESRD in ADPKD patients samples with

follow up of 13 years from Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) study population. The low-molecular weight urinary proteins were analyzed by CE-MS and used to generate a prognostic classifier (**Publication 1** [13]**)**. Based on the detected naturally occurring peptides (NOPs) produced during the process of proteolysis, we aimed to use several bioinformatics approaches to predict the proteases responsible for the generation of the urinary protein fragments. Additionally, investigation of the molecular pathways involved in ADPKD progression was attempted. Altogether, this scientific research of the surrogate biomarkers for ADPKD progression represent a crucial step for the clinical point of view and may potentially influence patient decisions about therapeutic intervention.

Subsequently, the next approach was designed to do an analysis of the urinary peptides by another analytical proteomic platform wildly used for biomarker assessment. Independently from CE-MS technology, MALDI-MS platform was evaluated for protein/peptide profiling and viewed as a promising tool for diagnosis of different diseases. Therefore, the second part (**Publication 2** [14]) was directed towards identification of new biomarkers allowing early and accurate detection of the septic AKI patients. Such biomarkers might initiate better and improved quality in the health care management of the AKI outcomes. In contrast to the single biomarkers who can not explain the complexity of the molecular processes characteristic for these septic AKI patients; previously developed CE-MS peptide marker pattern who showed a failure in point of care testing in clinical practice, performing a novel analysis with MALDI-MS-based platform to examine the abundance and reproducibility of peak intensity between cases and controls is highly important. The advantage of the new proteomic approach is challenging and clinically relevant.

Following the big scientific debate about the most appropriate fragmentation method applicable for identification of the natural occurring peptides (NOPs) by their amino acid sequences in clinical proteomics, the last part of this thesis (**Publication 3** [15]) was defined to investigate two complementary fragmentation approaches for retrieving high quality sequence information from human urine. Liquid chromatography (LC) coupled to higher-energy collision dissociation (HCD) and collision-induced dissociation (CID) MS instruments were extensively compared and examined. The human urinary peptide sequences with higher quality, better reproducibility and lower false discovery rate (FDR) were characteristic for HCD when compared to CID. In addition, the accuracy of each fragmentation method was attempted by evaluation of the possible erroneous

assignments. At this point, investigation of cysteine-containing peptides was performed since cysteine as an amino acid in her unmodified form can not be present in NOP and was considered as an artifact. Therefore, natural peptide characterization in urine elaborated in this research work without using complex sample preparation and enzymes with specific catalytic activity, emerges as a preferable method of choice in the state-of-art biomarker discovery.

2. Materials and Methods

2.1 Patient cohorts

The Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) cohort included in the first study consist of 241 patients with ADPKD. They were at the age between 15 and 45 years and had creatinine clearance >70 ml/min at baseline. All participants were followed with serum creatinine, iothalamate GFR, and MRI total kidney volume (TKV) measurement at baseline, year 1, 2, 3 (CRISP I), 6 and 8 (CRISP II). Clinical data of the majority patients were with 13 years of follow up. Randomly selected urine samples from 21 ADPKD patients with high-risk of ESRD and 39 ADPKD patients with a relatively stable function defined by mGFR and eGFR slope of not more than -3 ml/min/1,73m^2 year was examined in order to develop urinary peptide marker panel to predict progression towards renal failure. A detail description of the patients is given in Pejchinovski et al. [13], Grantham et al. [16], Chapman et al [17].

In the second study, measurements of the low molecular weight proteome for randomly selected 95 out of 195 septic AKI patients were performed. From the 95-patient pool, 17 patients with and 17 with out onset of AKI were randomly selected as a training group for statistical biomarker assessment and multimarker panel establishment. All participants were registered in a clinical trial and followed for one year in Ghent University Hospital. Conditions of sepsis, severe sepsis, and septic shock were determined according to American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference. Guidelines for Risk, Injury, Failure, Loss of kidney function and ESRD (RIFLE) based on serum creatinine and symptoms of urinary output were used to determine AKI patients after first 5 days of admission. The more extensive explanation can be found in Carrick et al. [14].

Evaluation of the sequence information was performed using pooled midstream morning urine sample from a healthy individual. A written consent of the participant was obtained and followed the guidelines of the Declaration of Helsinki [15].

2.2 Capillary electrophoresis coupled to mass spectrometry (CE-MS) analysis

The urine aliquots were stored at -80^{o}C and thawed immediately before the laboratory analysis. The volume of 700 µl was mixed with 700 µl solution containing 2M urea, 10mM NH4OH and 0,02% SDS. In the next step, aliquots were filtered with Centrisart

ultracentrifugation filters (20 kDa MWCO, Sartorius, Goettingen, Germany) to take out the higher molecular weight proteins. The rest of the filtrate was desalted using a PD 10 filtration column (GE Healthcare Bio Science, Uppsala, Sweden) to remove components of urea and electrolytes. Collected supernatants were lyophilized and stored on 4 ^{0}C. Re-suspension of the aliquots in 10µl HPLC grade H2O was used before CE-MS examination. CE-MS technology was performed using P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA), on-line couple to a Micro-TOF MS (Bruker Daltonic, Bremen, Germany). Spectra were accumulated every 3 s over m/z range of 350-3000 Da. Details of the CE-MS technology has been described previously by Mischak et al. [18].

2.3 Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis

Preparation of the urine volumes for MALDI-MS analysis was performed similarly like for CE-MS measurements [18]. The differences in the preparation protocol were serial dilutions required with the addition of 0.1% TFA. Exactly 12 dilution spots were placed on a 384 well MTP Anchorchip (Bruker Daltonics, Bremen, Germany) target plate in quadruplicate (25). To the volume of 1 µl dried sample, 5 mg/ml of the α-cyano-4-hydroxycinnamic acid (αCHCA) matrix (Laser Biolabs, 22 Sophia-Antipolis, France) was added. MALDI-MS laboratory analysis was performed using a Shimadzu Axima Confidence (Kratos, Manchester, UK) instrument in reflectron positive ion mode. In total, 50 laser shots and 36 MALDI-MS peptidomic profiles per sample were generated. For the peptide signals detection with a mass range from 100 to 4000 m/z, laser repetition rate was set to 50 Hz. The peak cleaning criteria were set to be: peak width of five, a Gaussian smoothing filter width of two and a baseline subtraction filter width of six. Detection peak method was 25% centroid with an arched threshold type, an offset of 0.2 mV and a 1-fold response factor. Additional information on the method is described by Carrick et al. [14].

2.4 Liquid chromatography coupled to mass spectrometry (LC-MS) analysis

LC-MS analysis of the same urinary liquid volume was carried out in five technical replicates for accurate comparison and evaluation of the performance of two different fragmentation methods. A Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK) was used to measure 5 µl of each urine sample. The aliquots were

loaded on a Dionex 0.1×20 mm 5 µm C18 nano trap column at a flowrate of 5 µl/min in 98% 0.1% formic acid and 2% acetonitrile. Later, they were processed by Acclaim PepMap C18 nano column 75 µm×15 cm, 2 µm 100 Å at a flow rate of 0.3 µl/min. To the column were added a gradient of solvent A:98% 0.1% formic acid, 2% acetonitrile verses solvent B: 80% acetonitrile, 20% 0.1% formic acid starting at 1% B for 5 minutes rising to 20% B after 90 min and finally to 40%B after 120 min. The ionization was performed with a Proxeon nano spray ESI source operating in positive ion mode into an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). The ionization voltage and capillary temperature were set at 2.6 kV and 250 °C. Further details on the whole methodology are provided by Pejchinovski et al. [15]

2.5 Proteomic data processing

CE-MS and LC-MS proteomic data processing were performed by usage of MosaVisu software package in order to deconvolve mass spectral ion peaks representing identical molecules at different charge states into single masses. By applying internal peptide standards normalization of migration time and ion signal intensity (amplitude) were achieved. Microsoft SQL database was generated to carry out the matching of all detected peptides with their molecular mass (kDa), CE-migration time (min) and ion signal intensity. For the MALDI-MS data, Shimadzu's MALDI-MS Launchpad v2.9.3 and IAMA softwares were used for examination of the peaks. Threshold of minimum 3 observed peaks and regression line was calculated per sample dilution. Only the peak data with negative regression slope were considered. Peptide marker panels were established using support vector machine (SVM)-based MosaCluster software package. Briefly, the MosaCluster logarithm uses Gaussian basis radial functions (RBF) as kernel function to map proteomic data into high dimensional feature space and create hyperplane. The role of the hyperplane is to separate the subject into two independent groups. More detail description can be found in Kistler et al. [19], Pejchinovski et al. [13], Mischak et al [20]. All further statistical analyses were done using MedCalc (version 12.7.5.0, MedCalcSoftware, Mariaakerke, Belgium; www.medcalc.be). Confidence intervals (95% CI) forsensitivity and specificity were estimated based on exact binomial calculation. The overall performance of the polypeptide pattern was evaluated by the receiver operating characteristic (ROC) area under the curve (AUC).

2.6 Sequencing data analysis

Mass spectrometer scanning modes were operated from 380 to 2000 amu. For each MS/MS method the top 20 multiply charged ions were selected for further investigation. ITMS analyser was used for CID-low MS/MS resolution mode with 60 000 resolution for MS1 and 1500 for MS2. FTMS analyser was used for HCD and CID-high resolution and set 60 000 resolution for MS1 and 7500 resolution for MS2. Peptides and proteins were searched against UniProt human database and identified with SEQUEST spectral algorithm (Thermo), without any enzyme specificity. Precursor mass tolerance and fragment mass tolerance were 10 ppm and 0.05 Da, respectively. No fixed modification was selected and oxidation of methionine and proline were set up as variable modifications. High confidence peptides with Xcorr ≥1.9,rank 1 and absence of unmodified cysteins were accepted as most valid for identification of the peptide markers as elaborated by Pejchinovski et al. [15]

2.7 Bioinformatic data analysis

Based on the peptide sequence information, a bioinformatics approach was attempted. Computational analysis for *in silico* proteases prediction with Proteasix was performed to identify proteases responsible for naturally occurring peptide generation. Additional information is provided by Klein et al. [21]

3. Results

3.1 Publication 1: Urinary proteome analysis predicts risk for end stage renal disease and reveals proteolytic pathways involved in autosomal dominant polycystic disease progression.

Patients from the CRISP cohort with a longitudinal follow up of 13 years were used to examine prognostic biomarkers in ADPKD. Out of 221 participants with available CE-MS data, 28 already reached ESRD. Only patients aged ≥ 24 at baseline were included given that no patients <24 years experienced ESRD during the follow up period. Initially, a subcohort of 81 patients was investigated. Two thirds of these patients with relatively stable kidney function and ESRD, were randomly chosen to create a development cohort for identification of the most altered urinary peptides. Remaining patients were used as validation cohort 1 (**Figure 1**). The most consistent biomarkers were selected by discarding 20% of cases and 30% of the controls and performing 10 permutations. This resulted in the identification of 20 significant peptide biomarkers between ESRD and controls, with an adjusted p-value of <0.05 and detected in >40% of the patients in at least one group (ESRD or control).

A SVM-based modelling was performed to develop a prognostic biomarker panel based on the identified 20 peptide markers. Applied to the development cohort (n=21 ESRD and 39 controls) after total cross validation, it reached an area under the receiver operating curve (AUC) of 0.95 to separate patients at low or high-risk for ESRD. When applied to validation cohort 1 (n=7 ESRD and 14 controls), AUC of 83 (p=0.0011, 95%-CI 0.60-0.96) was achieved (**Figure 1b**). In order to investigate the general utility of biomarker panel in clinical practice, we applied it to the entire CRISP cohort (n=142) with more than 10 years of follow up and older than 24 years at baseline, including intermediate progressors (validation cohort 2, **Figure 1b**). The ADPKD 20 biomarker panel achieved an AUC of 0.86 (95%-CI 0.79-0.91, p<0.0001) corresponding to an overall sensitivity of 86% and specificity of 71% at the predetermined cut-off level of -0.033. Based on these results, further approach has been attempted by comparing the prognostic value of the ADPKD 20 panel to that of htTKV as one of the best prognostic marker for diagnosis and prognosis of ADPKD. Interestingly, in validation cohort 2

htTKV achieved an AUC of 0.89 (95%-CI 0.83-0.94, p<0.0001) which was similar to the performance of ADPKD 20 (**Figure 1b**). It showed a sensitivity and a specificity of 82% and 84%, respectively, with a cut-off htTKV of >825 ml/m.

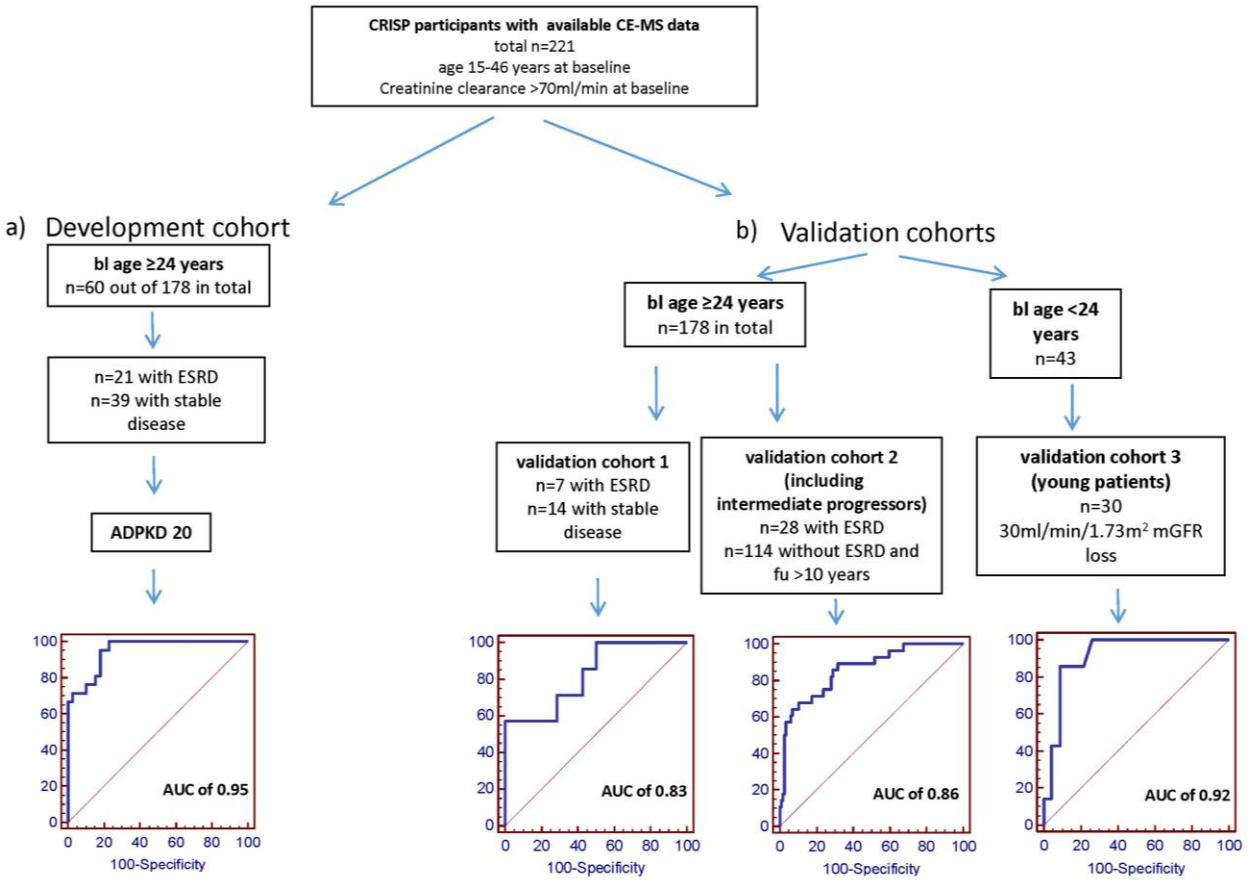

Figure 1. Schematic study flow chart of the patients selection and performance of the prognostic panel to discriminate patients progressing to ESRD during follow up from those with stable disease in (a) the development cohort consisting of 14 ESRD patients and 39 controls; (b) validation cohort 1 consisting 21 patients, validation cohort 2 with 142 patients and validation cohort 3 (young ADPKD patients) with 30 patients.

In addition, prediction of progression in young patients was assessed by applying ADPKD 20 panel to patients not older than 24 years of age and without ESRD as an outcome. Since they experienced a rapid decline of the renal function, a >30ml/min/1.73m^2 decline in mGFR from baseline to year 8 was used as an outcome measurement. When tested (**Figure 1b**), the prognostic biomarker panel achieved an AUC of 0.92 (95%-CI 0,76-0.99, p<0.0001).

Peptide ID	Sequence	Protein name	Protein accession	Amino acid start-stop position
99746	AGPpGApGAPGApGPVGPAGKSGDRGETGp	Collagen alpha-1(I) chain	P02452	1042-1071
30575	SpGSpGPDGKTGPp	Collagen alpha-1(I) chain	P02452	543-556
28132	TiSEKTSDQIH	Antithrombin-III	P01008	142-152
46928	EGSpGHPGQPGPpGPpG	Collagen alpha-1(III) chain	P02461	1175-1191
73015	ELTETGVEAAAAASAISVARTL	Plasma protease C1 inhibitor	P05155	448-468
68670	LLSPYSYSTTAVVTNPKE	Transthyretin	P02766	130-147
107929	DAHKSEVAHRFKDLGEENFKALVL	Serum albumin	P02768	25-48
22636	VGRHPLHLLE	Agrin	O00468	2044-2053
63517	EKETVIIPNEKSLQLQ	Mimecan	P20774	59-74
10581	EGTHSTKRG	Fibrinogen alpha chain	P02671	614-622
91421	MIEQNTKSPLFmGKVVNPTQK	Alpha-1-antitrypsin	P01009	398-418
90840	MIEQNTKSPLFMGKVVNPTQK	Alpha-1-antitrypsin	P01009	398-418
25893	DHEGTHSTKRG	Fibrinogen alpha chain	P02671	612-622
48699	IGPpGPAGApGDKGESGP	Collagen alpha-1(I) chain	P02452	769-786
51948	LSALEEYTKKLNTQ	Apolipoprotein A-I	P02647	254-267
40645	YTKKVPQVSTPTL	Serum albumin	P02768	435-447

Predicted proteases	Activity in ESRD / controls (mean±SEM)	p-Value
Cathepsin L*	-2.7 ± 0.9	0.008
Mmp2 **	3.9 ± 1.1	0.0001
Mmp3**	3.1 ± 0.8	0.0002
Mmp8**	3.5 ± 1.0	0.004
Mmp9**	-1.8 ± 0.8	0.036

Figure 2. Identified prognostic peptide markers with available sequence information and *in silico* predicted proteases that might be involved in ADPKD progression. All proteases marked with a star on this graph were previously reported to be found in ADPKD cyst fluid.

Lastly, NOPs from the CE-MS analysis were used to identify the specific proteases responsible for the generation of the urinary fragments included in the prognostic panel. Sixteen peptides were with available sequence information. *In silico* analysis was performed based on the mean intensities of the sequenced urinary peptides from the development cohort (ADPKD 20), protein accession number and cleavage amino acid positions. As a result, five proteases were predicted that displayed a significant correlation of their activity with progression to renal failure and were reported to be present in ADPKD cystic fluid (**Figure 2**). Collectively, the non-invasive urine-based test appears preferable alternative for clinical outcomes in ADPKD patients.

3.2 Publication 2: Development of a MALDI MS-based platform for early detection of acute kidney injury

In order to investigate the potential urinary biomarkers for discrimination of patients with acute kidney injury (AKI) from those patients without, MALDI MS-based platform was

assessed. Using this approach, 61 urine samples from sepsis patients with existing or developing AKI according to the Risk, Injury, Loss of kidney function and End stage renal disease (RIFLE) criteria and 34 from sepsis patients with preserved renal function were analyzed. Additionally, urine sample pools from normal controls, as well as from septic patients with or without AKI progression were defined and repetitively analyzed. Out of each group, 17 AKI cases and 17 non-AKI controls samples were randomly selected to develop a training cohort for statistical biomarker search and generation of the biomarker panel. From the statistical biomarker analysis, 39 out of the total 51 peptide markers were significant in Wilcoxon signed rank test after cross-validation and were selected for further examination.

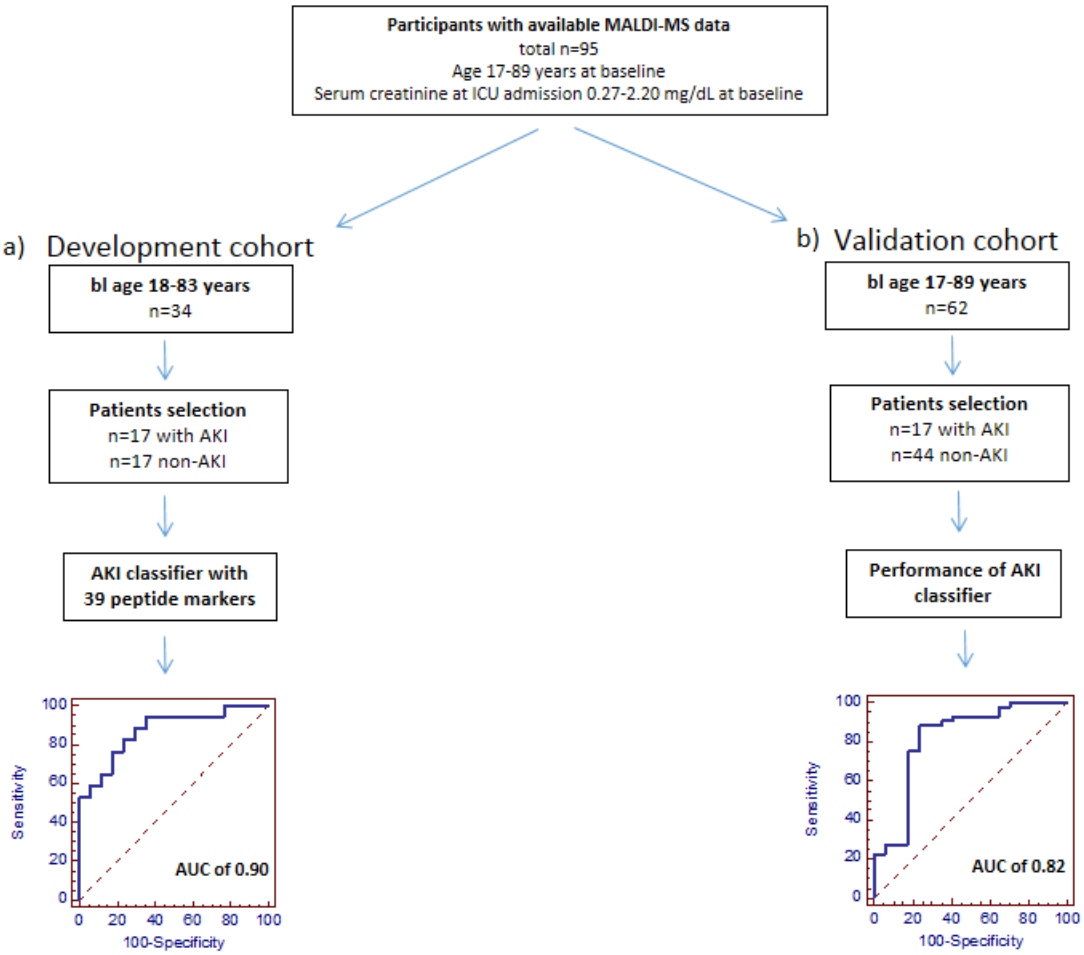

Figure 3. Schematic study flow chart of the patients selection and performance of the diagnostic panel to discriminate AKI cases from non-AKI control patients (a) the development cohort consisting of 17 AKI and 17 non-AKI patients and in (b) validation cohort consisting 17 AKI and 44 non-AKI patients.

Based on these 39 peptides that could be detected with low amplitude variability, an SVM-based classifier was developed to discriminate AKI from non-AKI patients. In this cohort, the biomarker panel achieved an AUC of 0.90 (95%-CI 0.77-0.97, p<0.0001). At a classification threshold of > -0.05, the biomarker model achieved a sensitivity 86% and a specificity 94% in the training cohort after total cross validation (**Figure 3a**).

Following the initial results, the diagnostic performance of the biomarker panel was subsequently applied to a validation cohort, consisting of remaining urine samples of 17 non-AKI patients and 44 AKI patients. Here, the biomarker panel achieved an AUC of 0.82 (95%-CI 0.70-0.91, p<0.0001). With the sensitivity of 86% and the specificity of 76% at the best threshold of -0.45, the biomarker panel just confirm its high capability for AKI detection (**Figure 3b**). To evaluate whether sepsis was a confounding factor or not for early detection of AKI, we applied MALDI-based biomarker panel on different sepsis stages (**Figure 4a**). The classification scores for the MALDI AKI marker were then plotted against their respective RIFLE scoring (**Figure 4b**). With the cut-off value of -0.05, the MALDI-based panel allowed to correctly predict the onset of AKI with significant differences between RIFLE stage 0 and 1 to 3.

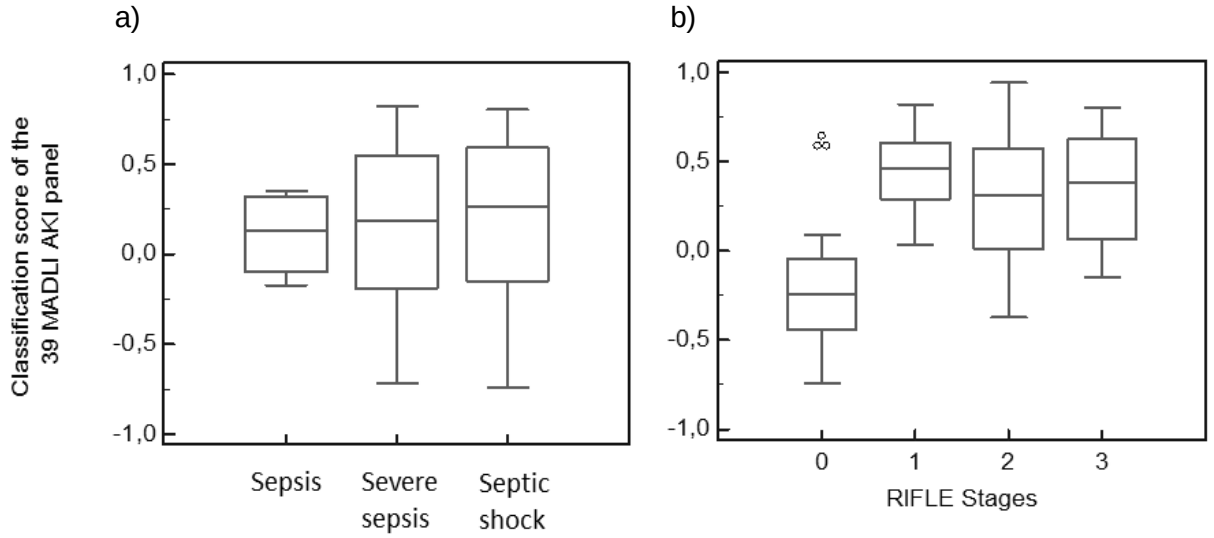

Figure 4. Classification of the 39 MALDI AKI panel in validation patient cohort (n=61). Distribution of the classification scores at cut off value of -0.05 in (a) group of patients with sepsis, severe sepsis and septic shock and (b) group of non-AKI patients (stage 0), at risk for AKI (stage 1), renal injury (stage 2) and renal failure (stage 3).

Peptide sequence information retrieved by MALD-MS/MS greatly improved the power of this methodology and continued to evolve peptide/protein identifications. However, more

accurate sequence identification was attempted by analyzing all AKI urine samples with CE-MS technology. This step was necessary to increase confidence in peptide matching and interpretation of the data. Therefore, from the 34 peptides included in MALDI biomarker panel, 9 could be detected by the CE and LC-MS fragmentation methods. Using CE-MS, we were able to retrieve 7 amino acid sequence information out of the 39 peptides included in the MALDI biomarker panel.

3.3 Publication 3: Comparison of higher-energy collisional dissociation and collision-induced dissociation MS/MS sequencing methods for identification of naturally occurring peptides in human urine

The large scale examination of the structure and function of the proteins has become one of the main interest in clinical proteomics and molecular medicine. An important aspect is now given to proteome analysis that is widely used for biomarker research to gain specific information that might discover the molecular mechanisms of complex biological processes. Up to now there were no reports for the optimal fragmentation methods of NOPs in human urine and they were omitted. To assess which of the MS/MS fragmentation methods is more suitable and more precise in the identification of the urinary amino acid sequences, five technical replicates of one standard urine sample were analyzed by LC-MS/MS with HCD, CID high and CID low resolution mass spec instruments. In addition, cysteine in the sequences has been used as a specific amino acid to recognize erroneous peptide assignments since, without any chemical treatment of the urine, free cysteines could not appear.

In the first part of the present study, the proteomic analysis was based on generating the high quality data set. This was done by restricting to peptide sequences that were at rank 1 and had Xcorr ≥1.9 as most valid to distinguish the best fragmentation performance. As a result, identification of total 770 sequences using HCD, 532 using CID High and 1499 using CID Low fragmentation methods were reported (**Figure 5a**). The average number of the peptide sequences identified per ran was higher for CID low when compared to HCD or CID high (421±27 versus 360±17 and 258±13). Similar results were observed when reproducibility of the total number of peptide sequences was examined. The analysis showed three times more sequences detected by CID low in contrast to HCD and CID high (1265 versus 367and 242).

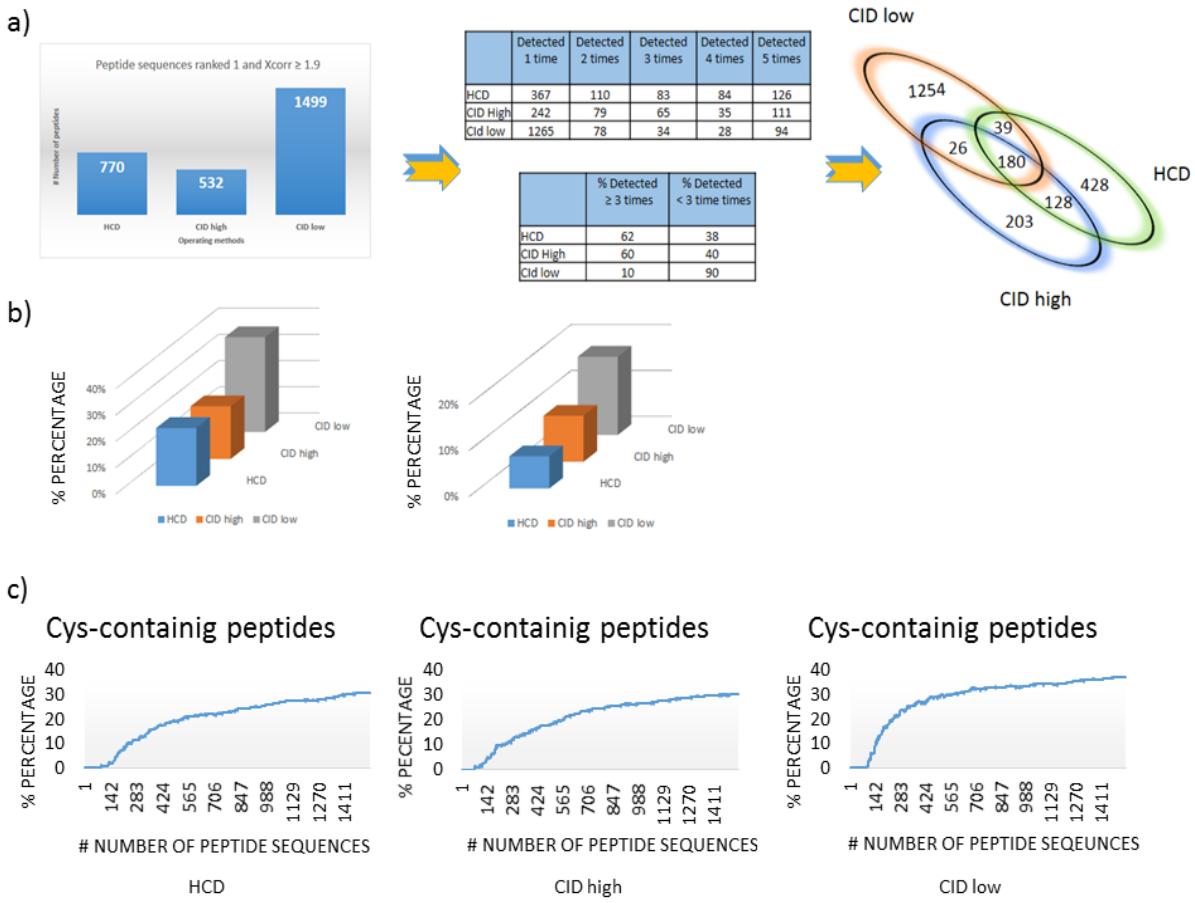

Figure 5. Standard urine sample analyzed by HCD, CID high and low resolution fragmentation methods. (a) Evaluation of total peptide sequences of rank 1 and Xcorr ≥ 1.9, their overlap and reproducibility. (b) Percentage of total urinary peptide sequences with cysteine ranked 1 with Xcorr ≥ 1.9 and their presence in the first 200 peptides with highest Xcorr. (c) Distribution of the cysteine-containing peptides in the in the first 1500 peptides ranked 1 and with highest Xcorr.

On the other hand, investigation the urinary peptide sequences detected repeatedly in at least three of the five replicates provide approximately 38% for HCD, 40% for CID high and 10% for CID low (**Figure 5a**). The sequences detected just once by CID low resolution were 90%.

In the further step, similarities in the performance of the three fragmentation modes were inspected by the number of common peptide sequences with rank 1 and Xcorr≥ 1.9. Overall there were 180 sequences identified by all MS/MS approaches (**Figure 5a**). The majority of them (123 sequences) were recognized by HCD and CID high while a small number of peptides were overlapping between CID low and HCD or CID high (39

and 26 sequences, respectively). Interestingly, it was found that more than 80% of unique peptide sequences were detected by CID low in contrast to other two approaches which displayed the much lower percentage of unique peptide sequences detection (55% for HCD and 35% for CID high).

Very important part of this study was a correct characterization of NOPs in human urine. Urinary peptide sequences that contain unmodified cysteine as amino acid considered to be inaccurate assignments. Results of the high quality peptide analysis (e.g sequences rank 1 and Xcorr≥ 1.9) displayed more than 10% less cys-containing peptides for HCD and CID high compared to CID low (23% for HCD, 21% for CID and 37% CID low, respectively) (**Figure 5a**). Similar results were achieved when analyzing the erroneous sequences assignment in the top 200 peptide sequences with highest Xcorr (7% for HCD, 10 % for CID high and 14% for CID low, respectively). In more detail examination, it was found that these so called artefacts were with lower reproducibility and more characteristic for CID low resolution mass spectrometer. Distribution of cys-containing peptides in the first 1500 sequences was further assessed (**figure 5c**). It was also reported more peptide sequences with cysteine detected by CID low in comparison to HCD and CID high (30% for HCD and CID high versus 36% for CID low). Similarly, FDR calculated by the classical methodology using Matrix Science perl script demonstrated two times more FDR for CID low compared to HDC and CID high (14% for CID low, 6.4% for HCD and 6.3% for CID high).

The main points of the proteomic results elaborated herein, allow both mass spectrometric strategies to measure low molecular weight proteins in human urine. The initial application of fragmentation methods on confirming the correct structure of peptides/proteins using different mass analyzers continues to be highly valued in clinical application. However, in this study, LC-MS/MS using HCD as optimized fragmentation method appears to be preferred when complete protein characterization is mandatory for improved productivity and efficiency in biomarker discovery.

4. Discussion

The successful and on time clinical diagnosis, prognosis and treatment decision continuously relies on the use of proteomic platforms that help in diseases detection and monitoring of the patient condition. By comprehensive analysis of the urine as a noble biological fluid which contains thousands of small peptides and proteins (<25kDa), in this dissertation, we aimed to investigate the current role of the urinary biomarkers in the prognosis of ADPKD progression and early diagnosis of AKI. This resulted in the development of two peptidomic patterns on different mass spectrometric techniques that can be potentially applied in clinical practice. In order to understand and translate the biological information coming from NOPs in urine, the most suitable fragmentation method to obtain amino acid sequence information was optimized.

Laboratory measurements of the urinary biomarkers are extremely important for every phase of disease development. Novel biomarkers allowing more accurate risk stratification are of crucial interest, to have overall health improvement of the patients. This is especially relevant in the case of ADPKD and AKI, where no signs or symptoms are manifested before renal dysfunction. The disease course of these renal complications remains to be with a high degree of variability among individuals. This especially affects the diagnostic and prognostic value of the peptide biomarkers, where age at ESRD would be of most clinical relevance. The typical signs and symptoms of kidney reduced filtration would require sufficient follow up period. GFR slope or a predefined GFR change not always assess the true disease state and are prone to inaccurate measurements influenced by the common medicaments given to the patients such as angiotensin-converting-enzyme inhibitors and angiotensin receptor blockers. Also, GFR is preserved and followed by glomerular hyperfiltration in the both disease, which causes accelerated decline towards renal failure. Although GFR measurement is known to be a “golden standard” or “overall index” of renal function, it did not outperform other markers for predicting renal outcomes who failed in clinical settings. Therefore, determination of GFR is not constant and difficult to predict.

After rapid expansion of the proteomics research filed in more than decade ago, MS-based technologies and their application becomes a fast growing field. To be in line with the novel strategies, our key point was to investigate the urinary peptidomics. As a

first step, clinically well-defined cohorts were used for identification of a urinary peptidomic biomarker panel that reliably predicts progression to ESRD in ADPKD patients. Here, ESRD was used as an outcome in patients aged ≥24 years at baseline, whereas a predefined GFR loss of 30 ml/min/1.73m^2 over 8 years, based on accurate iothalamate-based measurements, was used as an outcome in young ADPKD patients. To account for the dependence of follow up time and age at baseline, we restricted our analysis to CRISP participants with follow up time >10 years and age matched control group to ESRD cases. The CE-MS methodology used for urine peptidome analysis is standardized and available for clinical practice. Given this, there is no need to develop alternative analytical methods, such as specific ELISAs, to quantify the reported biomarkers. In comparison to TKV, which requires specific image algorithms not available for all clinical institutions, CE-MS has been used as an analytical tool for urine peptidome analysis with a high degree of reproducibility.

More detail investigation of the identified peptide panel provided mechanistic insights into molecular pathways of disease progression. To our knowledge, low molecular weight proteome/peptidome represents endogenous proteolytic cleavage fragments of larger proteins that are generated during proteolytic protein breakdown, modification, and turnover. Using this methodology, for 16 out of the 20 peptide markers included in the panel were identified. Five of the identified biomarkers were belonging to antithrombin III, fibrinogen alpha chain, alpha-1-antitrypsin and apolipoprotein A1 proteins which were previously reported to be present in the cystic fluid form ADPKD patients [22]. With the integration of bioinformatics tools, several proteases were predicted and confirmed by other studies that were responsible for the generation of these polypeptides closely related to ADPKD disease progression. The increased activity of matrix metalloproteases found in this study was indicative for the extracellular matrix remodeling during cyst expansion [23-25]. In addition, Alcalay et al. [26] reported decrease activity of cathepsin L in human ADPKD cells and murine Pkd null kidneys as a possible consequence of cyst enlargement. Collectively, the prognostic score of the 20 urinary peptide markers that allows stratification of ADPKD patients according to their risk to develop ESRD was superior to any other biochemical marker and appears preferable methodology for the clinics.

Following the advances in clinical proteomics, implementation of the MALDI-MS technology has been extensively proposed as a rapid screening approach in diagnosis and prognosis of a wide range of diseases. Albalat et al.[27] previously studied and

reported a method to improve the relative quantification of MALDI-MS analysis for proteomic biomarker assessment. Based on their findings, in this thesis, analytical method for faster and direct peptide analysis onto MALDI plate was explored. A simply applicable processing method was developed by usage a different range of sample dilutions placed on the MALDI plate and special algorithm for individual peptide quantification. At this point, MALDI software for quantitative analysis and automated data evaluation was developed. Moreover, for improved mass measurement of peptides due to lack of interference and issues with ion suppression CE was utilized prior to MS analysis. This well-developed strategy facilitated more comprehensive interrogation of the peptidomic datasets allowing novel peptide markers with highest biological interest to be selected. As a final step, biomarker panel with 39 polypeptides that accurately predict AKI in sepsis was established.

One of the goal during this scientific work was to provide the pathophysiological link with the complex processes occurring once AKI is present. Sequence information stored within the biomarkers helped us to identify 7 peptides that were bonded to the collagen alpha-1 (I) chain, collagen alpha-1 (II) chain, alpha-1-antitrypsin, beta-2-microglobulin and fibrinogen alpha chain. These findings were in accordance with the results from Metzger et al. [28], where 4 of them (collagen alpha-1 (I) chain, alpha-1-antitrypsin, beta-2-microglobulin and fibrinogen alpha) were reported to be associated with AKI and could be used as specific MALDI AKI biomarker “signature”. In terms of the different regulation of the proteins, observed prominent changes in the urinary collagen fragments in AKI compared to non-AKI sepsis patients have been already described to be linked with extracellular matrix deposition [29,30]. Similar findings were reported in other studies with renal complications [31-33]. Although, direct analysis of the urine as a biological sample via MALDI-MS technology for biomarker assessment has been limited due to non-quantitative nature of the technique, now, by the earlier presented methodology and software solution appear preferable option for the clinical laboratory.

The presented dissertation investigated another important aspect in complete characterization of the native polypeptides. Recently, Shen et al.[34] described several complementary fragmentation methods available for identification of naturally NOPs in human plasma, however, a study for sequencing of the urinary peptides was neglected. Therefore, methodological comparison of different fragmentation methods was proposed in order to determine the most appropriate approach in retrieving sequence information of the biomarker candidates. The presented data suggested HCD and CID

high resolution as more accurate sequencing methods in generating high quality data sets in comparison to CID low resolution. This is especially relevant for full characterization of NOPs in human urine where false-positive identifications should be minimum. Although CID low is widely used as fragmentation method for identification and quantification of proteins in complex samples, it displayed higher FDR (cysteine-containing peptides), less sensitivity and accuracy for matching of the urinary peptide sequences. Collectively, for urinary biomarker identification HCD or CID high resolution appear applicable for clinical proteomic research.

In conclusion, multidimensional urinary peptide panels identified throughout different mass spectrometric platforms carry the potential to improve the clinical diagnostic and prognostic value for many diseases. Additionally, obtaining correct peptide sequence information may provide a link to the pathophysiology of the underlying diseases and discover novel therapeutic targets for personalized healthcare of patients.

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6. Appendix

6.1 Affidavit

I, **Martin Pejchinovski** certify under penalty of perjury by my own signature that I have submitted the thesis on the topic **“The role of urinary peptide markers in diagnosis and prognosis of severe renal diseases”** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

_____

Date Signature

6.2 Statement of originality

Martin Pejchinovski had the following share in the following publications:

Publication 1: Pejchinovski M, Siwy J, Metzger J, Dakna M, Mischak H, Klein J, Jankowski V, Bae KT, Chapman AB, the CRISP investigators , Kistler AD. Urine peptidome analysis predicts risk for end stage renal disease and reveals proteolytic pathways involved in autosomal polycystic kidney disease progression. Nephrol Dial Transplant. 2016

Contribution in detail: Performed the experiments and interpreted the data, contributed reagents/materials/analytical tools, wrote and revised the manuscript.

Publication 2: Carrick E, Vanmassenhove J, Glorieux G, Metzger J, Dakna M, Pejchinovski M, Jankowski V, Mansoorian B, Husi H, Mullen W, Mischak H, Vanholder R,Van Biesen W. Development of a MALD MS-based platform for early detection of acute kidney injury. Proteomics Clin Appl. 2016

Contribution in detail: Performed sequence peptide identifications, wrote the manuscript, contributed in the revision of the manuscript and critically evaluated the manuscript.

Publication 3: Pejchinovski M, Klein J, Ramírez-Torres A, Bitsika V, Mermelekas G, Vlahou A, Mullen W, Mischak H, Jankowski V. Comparison of higher-energy collisional dissociation and collision-induced dissociation MS/MS sequencing methods for identification of naturally occurring peptides in human urine. Proteomics Clin Appl. 2015

Contribution in detail: Design the experiments, process and interpreted the data, wrote and revised the manuscript.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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6.3 Selected publications

Original article 1:

Pejchinovski M, Siwy J, Metzger J, Dakna M, Mischak H, Klein J, Jankowski V, Bae KT, Chapman AB, theCRISP investigators, Kistler AD. Urine peptidome analysis predicts risk for end stage renal disease and reveals proteolytic pathways involved in autosomal dominant polycystic kidney disease progression. Nephrol Dial Transplant 2016. first published online July 5, 2016

DOI:10.1093/ndt/gfw243

Original article 2:

Carrick E, Vanmassenhove J, Glorieux G, Metzger J, Dakna M, Pejchinovski M, Jankowski V, Mansoorian B, Husi H, Mullen W, Mischak H, Vanholder R, Van BW. Development of a MALDI MS-based platform for early detection of acute kidney injury. Proteomics Clin Appl 2016. first published online July 10, 2016

DOI:10.1002/prca.201500117

Original article 3:

Pejchinovski M, Klein J, Ramirez-Torres A, Bitsika V, Mermelekas G, Vlahou A, Mullen W, Mischak H, Jankowski V. Comparison of higher energy collisional dissociation and collision-induced dissociation MS/MS sequencing methods for identification of naturally occurring peptides in human urine. Proteomics Clin Appl 2015. First published online June 5, 2015

DOI:10.1002/prca.201400163

My curriculum vitae does not appear in the electronic version of my paper for reason of data protection.

List of publications:

- Original articles:

1) Pejchinovski M, Siwy J, Metzger J, Dakna M, Mischak H, Klein J, Jankowski V, Bae KT, Chapman AB, theCRISP investigators, Kistler AD. Urine peptidome analysis predicts risk for end stage renal disease and reveals proteolytic pathways involved in autosomal dominant polycystic kidney disease progression. Nephrol Dial Transplant 2016; in press.

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6.6 Acknowledgements:

First and foremost, I would like to thank my supervisors Prof. Dr. Harald Mischak, Dr. Justyna Siwy and Dr. Jochen Metzger for selecting me to be one of the students and to join Mosaiques Diagnostics GmbH. Thank you very much for teaching and guiding me during all these three years. This was very helpful for completing my Doctoral studies which were one of the reasons for being in Hannover. You made me stronger, dedicated and successful scientist.

Special gratitude to PD Dr. Vera Jankowski who gave all the support in guidance and mentorship of my research work.

I would also like to thank all the people from the TranCYST project and Marie Cure fellowship for their parental-care during our meeting and conferences. We really had a great time!

Particular thanks go to all students, scientists and lab people from Mosaiques. I would not forget the precious time speeded together and the funny discussions as part of my stay in Hannover.

Special gratitude to Dr. Andreas Kistler and Dr. Arlene Chapman for their assistance, mentorship, fruitful discussions and willingness to teach me how to proceed with my research projects. I will always remember your support and motivation not just during this time period but also on the conferences that I participated. You are real professionals!

Lastly, I would like to thank my family for their supportive attitude during all three years. You were so encouraging form day one and really appreciate all the motivation given to me whole my life. I owe my success to you. On the end we made it, we are one step forward!!! And most of all I would like to thank my lovely, precious, patient and encouraging girlfriend Ilinka who was with me all the time. Your supportive and caring attitude help me in my self-confidence. Love you so much!!!