Aus dem Institut/der Klinik für Nephrologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Clinical peptidomics for non-invasive diagnosis and prognosis of renal diseases

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

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von
Claudia Pontillo

aus Napoli, Italy

Datum der Promotion: 25.06.2017

Table of contents

List of Abbreviations

A1AT-α-1-antitrypsin; **CE-MS**-Capillary electrophoresis coupled to mass spectrometry; **CKD**-Chronic kidney disease; **DN**-diabetic nephropathy; **eGFR**-Estimated glomerular filtration rate; **ESRD**-End stage renal disease; **FSG**-focal segmental glomerulosclerosis; **GN**-glomerulonephritis; **IgAN**-IgA nephropathy; **LN**-lupus nephritis; **MALDI-MSI**-matrix-assisted laser desorption ionization imaging mass spectrometry imaging; **MCD**-minimal-change disease; **MGN**-membranous glomerulonephritis; **MN**-membranous nephropathy; **N**-nephrosclerosis; **RAS**-renin-angiotensin system; **SD**-standard deviation; **UAE**-urinary albumin excretion; **Vasculitis**-vasculitis-induced kidney disease

Abstract

Capillary electrophoresis coupled to mass-spectrometry (CE-MS) and matrix-assisted laser desorption ionization imaging mass-spectrometry (MALDI-MSI) emerged as highly reproducible techniques to discover potential naturally occurring peptide biomarkers in urine and fresh-frozen renal tissue samples. These biomarkers were established for the non-invasive diagnosis and prognosis of renal diseases.

The first part of the PhD thesis in investigation of the performance of an established urinary proteome-based classifier ("CKD273") depicting progression in different CKD stages and the relevance of changes in individual urinary peptides for the pathophysiology of CKD by the analysis of urine proteome profiles. Out of 2,672 samples included in the study, 394 individuals displayed a decline in eGFR of >5ml/min/1.73m^2 per year (defined as progressively diseased). In early CKD stages the "CKD273"-classifier performed significantly better than the clinical marker "albuminuria" in detecting progressors, while albuminuria performed better in patients at late stages of CKD. At early CKD stages, collagen fragments were highly excreted in urine, while at moderate and late stage of CKD, markers of inflammatory process and functional loss (such as alpha-1-antitrypsin, CD99 antigen, antitrombin III, serum albumin etc.) were more abundant.

Secondly, MALDI-MSI was applied to bioptic renal tissue from patients with focal segmental glomerulosclerosis (n=6), IgA nephropathy, (n=6) and membranous glomerulonephritis (n=7), and from controls (n=4) to define molecular signatures of primary glomerulonephritis. Signal of m/z 4,048 present in MALDI-MS imaging was identified as α-1-antitrypsin (A1AT). By immunohistochemistry it was shown that A1AT was localized to the podocytes within sclerotic glomeruli. This suggested that A1AT could be a marker of podocyte-stress correlating with development of focal segmental glomerulosclerosis. Additionally, correlation of MALDI-MSI findings with CE-MS findings of urinary peptidomics identified the same A1AT peptide as up-regulated in CKD patients.

In the final part of the thesis assessment of urinary biomarkers for different aetiologies of CKD was investigated by the identification of peptides contained in 1,180 urine samples analysed via CE-MS. For 7 type of CKDs, potential biomarkers were defined and combined into classifiers. Validation of these classifiers in an independent cohort showed accuracy ranging from 0.77 to 0.95 for discrimination of one CKD etiology from the other. Sequence analysis of the biomarkers provided link to pathophysiology of CKD.

Abstract in German

Die direkt mit einer Flugzeit-Massenspektrometrie (CE-MS) gekoppelte Kapillarelektrophorese sowie die bildgebende matrix-unterstützte Laser Desorption/Ionisation Massenspektrometrie (MALDI-IMS) haben sich als hoch-reproduzierbare Techniken zur Identifizierung von potentiellen natürlich vorkommenden Peptidbiomarkern in Urin beziehungsweise Nierengewebeproben erwiesen. Diese Biomarker wurden für die nicht-invasive Diagnose und Prognose von Nierenerkrankungen etabliert.

Im ersten Teil der Dissertation habe ich die Leistung des auf dem Urinproteom-basierenden Biomarkermodells "CKD273" untersucht, das zur Klassifizierung von chronischen Nierenerkrankungen (CKD) etabliert worden ist. Dabei wurde die Vorhersage der Progression der CKD in verschiedenen Krankheitsstadien durch die Klassifikation von Urinproteomprofilen beurteilt. Weiterhin wurde die Bedeutung von Veränderungen einzelner Peptide für das Verständnis des pathophysiologischen Mechanismus der CKD Progression beurteilt. Für diese Beurteilungen wurde das Urinproteom von 2.672 Patienten mit verschiedenen CKD Stadien untersucht. Von diesen Patienten zeigten 394 einen Rückgang der geschätzten glomerularen Filtrationsrate (eGFR) von >5 ml/min/1,73m^2 pro Jahr und wurden daher als progressiv Erkrankte eingestuft. In frühen CKD-Stadien zeigte der "CKD273"-Klassifier im Vergleich zum bekannten klinischen Marker Albuminurie eine bessere prognostische Leistung bei der Identifikation von progressiv Erkrankten. Im Gegensatz dazu zeigte in späten Stadien der CKD die Albuminurie die bessere prognostische Leistung. Darüber hinaus lieferte die Entdeckung einer erhöhten Ausscheidungsrate von Kollagenfragmenten im Urin in einem frühen Stadium der CKD und von Markern entzündlicher Prozesse und eines Funktionsverlusts (z.B. alpha-1-Antitrypsin, Antithrombin III, Serumalbumin, usw.) Hinweise auf den pathophysiologischen Mechanismus der Progression.

Im zweiten Teil der Dissertation wurde MALDI-IMS auf Nierengewebebiopsien angewandt, um molekulare Signaturen einer primären Glomerulonephritis zu definieren. Die Biopsien stammten von Patienten mit einer fokal segmentalen Glomerulosklerose, IgA-Nephropathie, und membranöser Glomerulonephritis sowie von gesunden Kontroll-Probanden. Ein Massenpeak mit einem m/z-Verhältnis von 4.048 wurde als α-1-Antitrypsin (A1AT) identifiziert. Die Immunhistochemie zeigte, dass das α-1-Antitrypsin in Podozyten der sklerotischen Glomeruli lokalisiert ist. α-1-Antitrypsin kann somit als Marker für zellulären Stress der Podozyten bei der

Entwicklung einer fokalen segmentalen Glomerulosklerose dienen. CE-MS-basierende peptidomische Untersuchungen bestätigten die verstärkte Ausscheidung desselben A1AT Peptidfragments im Urin von CKD-Patienten und somit die MALDI-IMS Ergebnisse.

Im dritten Teil der Dissertation sollten spezifische Biomarker für verschiedene Ätiologien der CKD identifiziert werden. Um dieses Ziel zu erreichen, analysierte ich die auf CE-MS basierenden Peptidome von 1.180 Urinproben. Für sieben verschiedene CKD Ätiologien, der Unterscheidung von CKD Ätiologien Messgenauigkeiten von 0,77-0,95. Dies zeigte, dass man mittels Urinpeptidomanalyse zwischen verschiedenen CKD-Ätiologien unterscheiden kann. Die Sequenzanalyse der Biomarker ergab zudem Erkenntnisse zur Pathophysiologie der CKD Ätiologien.

1. Introduction

In the post genomic era, the concept of peptidomics refers to the low molecular weight biomolecules, ranging from 0.5 to 15 kDa [1] and aims at the comprehensive network of small polypeptides as link between proteins and metabolites. Peptides are considered as biological molecules such as cytokines, growth factors, inhibitors, activators or substrates of a pathway, hormones, and neuropeptides [2;3] originated from larger precursors and involved directly or indirectly in the communication between cells. Peptides are small molecules derived from *in vivo* cleaved enzymes resulting as the products of natural proteolysis. Particularly, different concentrations of peptides among compartments (such as tissue, cells, and body fluids) alter their diffusion or secretion to other compartments reflecting specific biological processes.

Since the last decade, advanced mass-spectrometric technologies allow analysis and identification of proteins and peptides. This contributes to the emerging of clinical proteomics and peptidomics to be used as diagnostic and prognostic tools for several diseases. In particular, capillary electrophoresis coupled to mass-spectrometry (CE-MS) [4] and MALDI-MS imaging (MALDI-MSI) [5] have been emerged as high reproducible techniques analysing naturally occurring peptides in urine samples and fresh-frozen renal tissue, respectively. Currently, these techniques are highly applicable for discovery and validation of potential peptide-based biomarkers of renal diseases. For instance, in the year 2010 Good et al. [6] used CE-MS to develop a classifier composed of 273 urinary peptides ("CKD273"-classifier) to diagnosis of chronic kidney disease (CKD). The study included 379 healthy subjects and 230 patients suffering from various kidney diseases. The validity of the "CKD273"-classifier was confirmed in several studies for diagnosis and prognosis of CKD [7-10].

CKD is defined as a heterogeneous disease generally characterized by abnormalities in kidney structure and function lasting for more than three months [11]. Between the 1988-1994 and the 2003-2006, the National Health and Nutrition Examination Survey and National Health and Nutrition Examination Survey studies reported that the prevalence of CKD in people aged 60 years and older increased from 18.8 to 24.5%, whereas for people between age of 20–39 years, it remained below 0.5%. The definition of a specific type of kidney disease is dependent on genetic factors, environmental influences and/or demographical characteristics. Several types of CKDs, such as focal segmental glomerulosclerosis, IgA nephropathy, membranous glomerulonephritis, minimal-change disease, membranous nephropathy, diabetic nephropathy, nephrosclerosis, lupus nephritis and vasculitis-induced kidney disease, are presently diagnosed by kidney biopsies.

However, due to the invasiveness of this method, alternative procedures to define several CKDs are required.

CKD progression is detected using marker of kidney function such as assessing decline in estimated glomerular filtration rate (eGFR) [12] and/or increase of urinary albumin excretion [13]. According to guidelines of "Clinical Practice Guideline for Diabetes and CKD" (KDOQI 2012)[11], on the basis of the eGFR, CKD is classified into five stages: stage 1 (eGFR>90 ml/min/1.73 m^2), stage 2 (eGFR 60–89 ml/ min/1.73 m^2), stage 3 (eGFR 30–59 ml/min/1.73 m^2), stage 4 (eGFR 15–29 ml/min/1.73 m^2) and stage 5 (eGFR<15 ml/min/1.73 m^2). Based on urinary albumin excretion, three CKD-stages are defined: stage 1 (albuminuria<30mg/24h) (normoalbuminuria), stage 2 (albuminuria>30mg/24h) (microalbuminuria) and stage 3 (albuminuria>300mg/24h) (macroalbuminuria) [11].

Despite the clinical performance of these markers, albuminuria sometimes does not display high accuracy for the selection of individuals at high risk that would benefit most from timely therapeutic interventions [14] [15] [16] [17]. A significant reduction of eGFR is associated with structural kidney damage. However, eGFR is considered to be a marker of advanced disease [18], when clinical guidance could be limited.

Therefore, there is an urgent need for new biomarkers detecting pre-clinical pathophysiological alterations at an early stage of the disease to guide individual therapeutic interventions for prevention of disease progression. Following this, the first part of the PhD thesis (**Publication 1**) was focused on investigation of the performance of a previous established urinary proteome classifier named "CKD273"-classifier compared to urinary albumin excretion (UAE) in predicting the progression of CKD at different disease stages. Changes in individual urinary peptides for each stage of disease were also investigated for improved understanding of pathophysiology of CKD using the urinary proteome data of 2,672 CKD patients. The second part of the thesis referred to **Publication 2** was focused on the possibility to assess different aetiologies of CKD, such as membranous glomerulonephritis (MGN), focal segmental glomerulosclerosis (FSGS), and IgA nephropathy by the identification of peptides and proteins contained in bioptic fresh frozen renal tissue analysed via MALDI-MS imaging. Potential urinary biomarkers for membranous glomerulonephritis (MGN), focal segmental glomerulosclerosis (FSGS), and IgA nephropathy, minimal-change disease (MCD), membranous nephropathy (MN), diabetic nephropathy (DN), hypertensive nephrosclerosis (N), lupus nephritis (LN) and vasculitis-induced kidney disease (vasculitis) were also defined in **Publication 3** using the urinary proteome data of 1,180 patients displayed the aforementioned CKD etiologies.

2. Methods

All detailed information on samples preparation, collection, and analysis are reported in **Publication 1**, **2** and **3,** respectively.

2.1 Patient cohort and sample preparation

For prognosis of CKD and for differential diagnosis of CKD assessment, all of the CKD patient data were extracted from the Human Urinary Proteome database [19;20]. Stages of CKD were defined based on eGFR calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [21]. For prognosis of CKD 2,672 data of patients suffering from CKD were used. For diffential diagnosis, data of patients with focal segmental glomerulosclerosis (FSGS, n=110, different variants), IgA-nephropathy (IgAN, n=179), minimal-change disease (MCD, n=35), membranous nephropathy (MN, n=77), diabetic nephropathy and nephrosclerosis (DN&N, n=576), lupus nephritis (LN, n=92) and vasculitis-induced kidney disease (n=111) were included. A subset of datasets of 706 samples was used for discovery and a separate group of 474 samples was used for validation. In total 1,180 data were used.

For tissue analysis, the MALDI-imaging tissue study included only primary glomerulonephritis classified as idiopathic (GN) according to clinical criteria. Fresh-frozen biopsies taken from patients, who underwent renal biopsy with a histological diagnosis of focal segmental glomerulosclerosis (FSGS, n=6), IgA Nephropathy (IgAN, n=6) and membranous glomerulonephritis (MGN, n=7), were collected. Normal cortical biopsies (controls, n=4) corresponded to regions of kidney obtained from radical nephrectomy during tumor treatment. Control patients had no history of functional renal disease. Sample collection and preparation of urine samples were addressed as in the study by Good et al. [6]. Sample collection and preparation of renal tissues were addressed as in the study by Mainini et al. [5].

2.2 CE-MS and MALDI-MSI analysis

CE-MS analysis a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) was coupled with a Micro-TOF MS (Bruker-Daltonic, Bremen, Germany). All additional details are reported in [22;23]. For normalization caused by analytical variances and differences in urine dilution, MS signal intensities were normalized relative to 29 internal standard peptides

generally present in at least 90% of all urine samples with small relative standard deviation (SD) [24].

For MALDI-MSI analysis of tissue samples, all mass spectra were acquired in linear positive mode in the mass range of 3,000 to 20,000 m/z using an UltrafleXtreme (Bruker Daltonic Bremen, Germany) External calibration was performed using a mixture of standard proteins within the mass range of 5,500 to 17,000 m/z (ProtMix I, Bruker-Daltonic Bremen, Germany).

2.3 MS/MS analysis sequences

MS/MS mass spectra were acquired in positive ion mode using an Orbitrap Velos FTMS (Thermo-Finnigan, Bremen, Germany) as described by Zürbig et al. [25]. Data files were searched against the UniProt human non-redundant database using Proteome Discoverer 1.2 (Thermo-Finnigan, Bremen, Germany), using the SEQUEST database. Relevant settings were: no fixed modifications allowed, oxidation of methionine and proline were allowed as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass of 6,000 Da with a minimum peak count of 10. The high confident peptides were defined by cross-correlation (Xcorr) ≥1.9. Precursor mass tolerance was 10 ppm and fragment mass tolerance was 0.05 Da. For MALDI-MS/MS, representative mass spectra were acquired in reflectron positive mode in the mass range of 700 to 4,500 m/z. Precursor ions were selected and fragmented using the laser-induced dissociation (LID) and an MS/MS spectra obtained from the accumulation of ~10,0000 laser shots. External calibration was performed using a mixture of standard peptides within the mass range of 750 to 3,500 m/z (PepMix I, Bruker Daltonics, Bremen, Germany). All MS/MS spectra were searched against the Swiss-Prot database with the Mascot 2.4 search engine (Matrix Science, London, UK).

2.4 “CKD273”-classifier analysis and statistical analysis

The “CKD273”-classifier is an SVM-based classification model, where classification is performed by determining the Euclidian distance (defined as the SVM classification score) of the 273-dimensional vector to a 272-dimensional maximal margin hyperplane, as previously described by Good et al. [6] Rapid progression was defined as described in the “Kidney Disease Improving Global Outcomes 2012” CKD guidelines as a sustained decline in eGFR of 5 ml/min per 1.73 m^2 per year or more [11]. To assess the value of urinary albumin excretion (mg/24h) and CKD273 at different disease stages, the cohort was stratified by baseline eGFR (Chronic Kidney Disease in Epidemiology Collaboration formula) [21] ≥80, 79-70, 69-60, 59-50, 49-40, 39-30, and <29

mL/min/1.73m^2. The sensitivity, specificity, Box-Whisker, logistic regression and area under the curve (AUC) were calculated using ROC analysis with MedCalc software (version 12.1.0.0, http://www.medcalc.be; MedCalc Software, Mariakerke, Belgium). For biomarker discovery, non-parametric Wilcoxon rank sum test was used to calculate the p-values of individual peptides. The false discovery rate adjustments of Benjamini-Hochberg were employed to correct for multiple testing [26]. An adjusted P-value of 0.05 was selected as significance level.

2.5 Immunohistochemistry (IHC) with alpha-1-antitrypsin (A1AT) antibody

For tissue samples analysis, a series of formalin-fixed paraffin-embedded (FFPE) renal biopsies taken from patients with IgAN and FSGS were tested for IHC analysis. This validation group included IgAN with wide sclerosis (n=4), IgAN with a prevalent mesangioproliferative pattern without sclerosis (n=4) and FSGS (n=6). For each specimen, 3μm thick sections were cut from FFPE blocks. After deparaffinization and rehydration, slides underwent endogenous peroxidase blockage and an antigen retrieval process via EnVision Flex (Dako, Glostrup, Denmark). Finally, Autostainer Link 48 (Dako, Glostrup, Denmark) was used to apply the primary antibody directed against A1AT (polyclonal rabbit, Dako, Glostrup, Denmark).

3. Results

3.1 Publication 1: Pontillo C, Lotte Jacobs, Staessen JA, Schanstra JP, Rossing P, Heerspink HJL, Siwy J, Mullen W, Vlahou A, Mischak H, Vanholder R, Zürbig P, Jankowski J. A urinary proteome-based classifier for the early detection of decline in glomerular filtration. Nephrol Dial Transplant. 2016 Jul 6. pii: gfw239

Firstly, we investigated the possibility of assessment of CKD progression at different stages of the disease by the use of urinary-based classifier "CKD273"[6] compared to clinical parameter of urinary albumin extraction rate (UAE). The study included 2,672 subjects with mean (±SD) follow up time of 3.3±1.1. Peptide-profiles of those patients were obtained via CE-MS and extracted from Human Proteome Database [19;20]. Out of 2,672 subjects, 394 were classified as progressors towards CKD displaying an eGFR decline>5mL/min/1.73m^2 per year [11]. The remaining 2,278 subjects were defined as non-progressors during the follow up time (eGFR decline<5mL/min/1.73m^2 per year). According to "The National Kidney foundation"[11], CKD can be defined by five stages based on eGFR. When stratification of the patients in the current cohort according to the official CKD stages was applied, we observed a discrepancy in the distribution of the patients (59.6% of the subjects was in CKD stage 2). Therefore, in order to obtain a stronger homogeneity of the patients and higher resolution for the assessment of CKD stages, it was adopted a different method to stratify the patients, according to the following eGFR strata: >80, 79-70, 69-60, 59-50, 49-40, 39-30, and <29 mL/min/1.73m^2. With this stratification the group with the highest number of patients (eGFR>80 ml/min/1.73m^2) represented 37.1% and the group with the lowest number of patients (eGFR 40-49 ml/min/1.73m^2) represented 3.6% of the total cohort. Although the proportion of patients per group was not identical, its distribution was significantly improved compared to using classical CKD stages. As next step, we compared UAE and CKD273-classifier for the prognosis of progression of CKD in each eGFR strata. Progressors/cases were defined as having a decline in eGFR of >5ml/min/1.73m^2 per year [11]. In the groups of patients with eGFR >80 and 70-79 ml/min/1.73m2 the proteome-based classifier displayed an AUC significantly higher (P<0.005) than albuminuria. In the groups of patients with eGFR between 60-69 and 50-59 ml/min/1.73m2 the AUCs of the two markers had no significant differences (P>0.005). At late CKD stages (eGFR ranging from 40-49 and <29 ml/min/1.73m^2), albuminuria was significantly better than "CKD273"-classifier, but not in the group of patients with eGFR 30-39 ml/min/1.73m^2 where the proteome-based classifier displayed again similar performance than albuminuria (P>0.005). These results are graphically depicted in **Figure 1**.

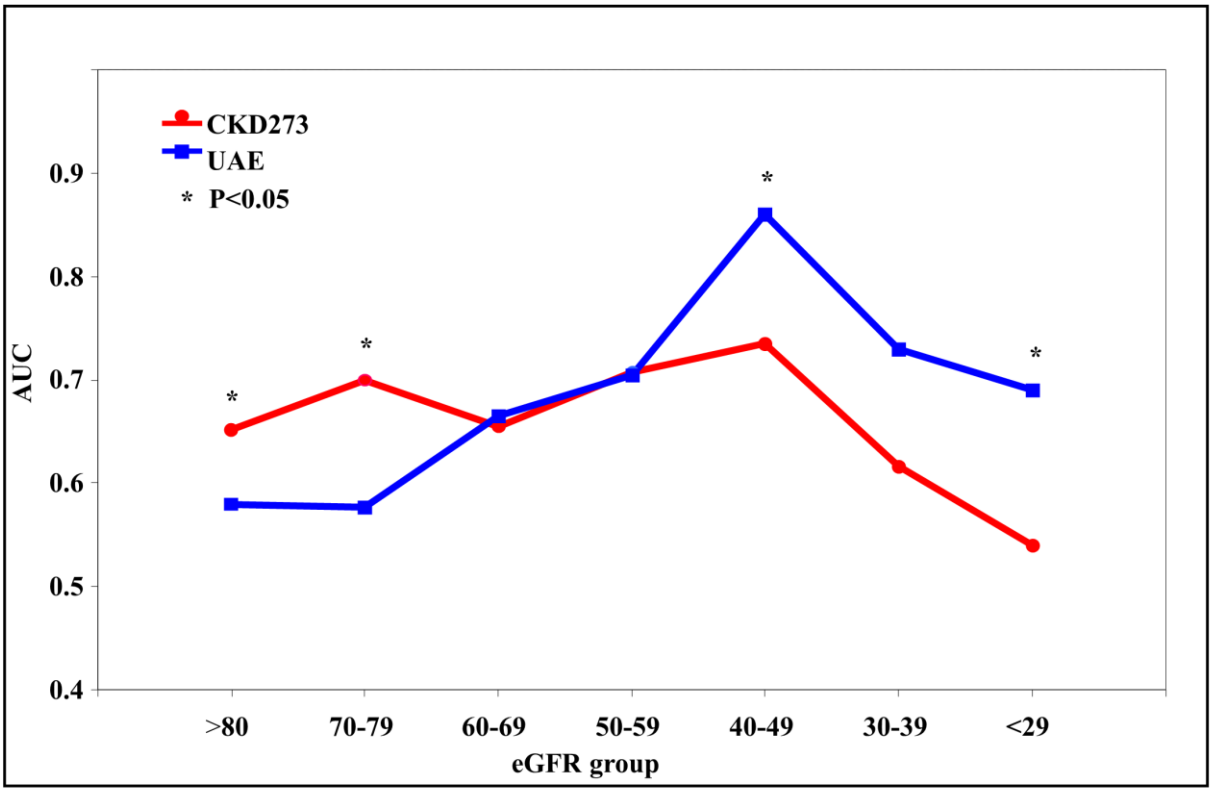

Figure 1. Comparison of the ROC-analyses for prognosis of CKD at in each eGFR stratum. The "CKD273"-classifier (red curve) performs better at early CKD stages, while UAE (blue curve) has higher AUCs at late CKD stages.

In our cohort, the variability of the albuminuria values irrespective of the patients being progressors or not and irrespective of the eGFR stratum, was higher than the "CKD273" scores. In addition, the "CKD273" scores were gradually increasing with decreasing eGFR, while the values of albuminuria were increased in the moderately advanced stages for progressors. Secondly, individual urinary peptides not present in the classifier were investigated in relation to CKD progression for underlying possible mechanisms of disease-pathophysiology. Due to the high complexity of CKD as heterogeneous disease, many urinary peptides can be associated with different eGFR strata. Using the non-parametric Wilcoxon test, we performed *de novo* statistical analysis to identify which urinary peptides were associated with single eGFR strata. In order to avoid introducing bias among the seven eGFR groups (due to the different number of patients present in each group) we focused on the ten most significant peptides based on significance. As reported in **Figure 2**, the results of the analysis suggested the presence of peptides associated to three types of protein function: structural changes (red colour), inflammatory process (green

colour) and loss of kidney function (blue colour). At early stages of CKD (eGFR 70 to ≥80 ml/min/1.73m^2), several collagen fragments were reported as the most abundant peptides found in the urine of patients. At moderately advanced disease (eGFR 40 to 69 ml/min/1.73m^2), peptides of inflammatory processes derived from alpha-1-antitrypsin, CD99 antigen, antitrombin III, polymeric immunoglobulin receptor, basement membrane-specific heparan sulfate proteoglycan core protein, Ig gamma-1 chain C region, kininogen-1 proteins were reported. In the late CKD stages (eGFR <39ml/min/1.73m^2) fragments derived from serum albumin, alpha-2-HS-glycoprotein, beta-2-microglobulin, vitamin D-binding protein, serum amyloid A-1 protein were present in urine of the subjects used in the study.

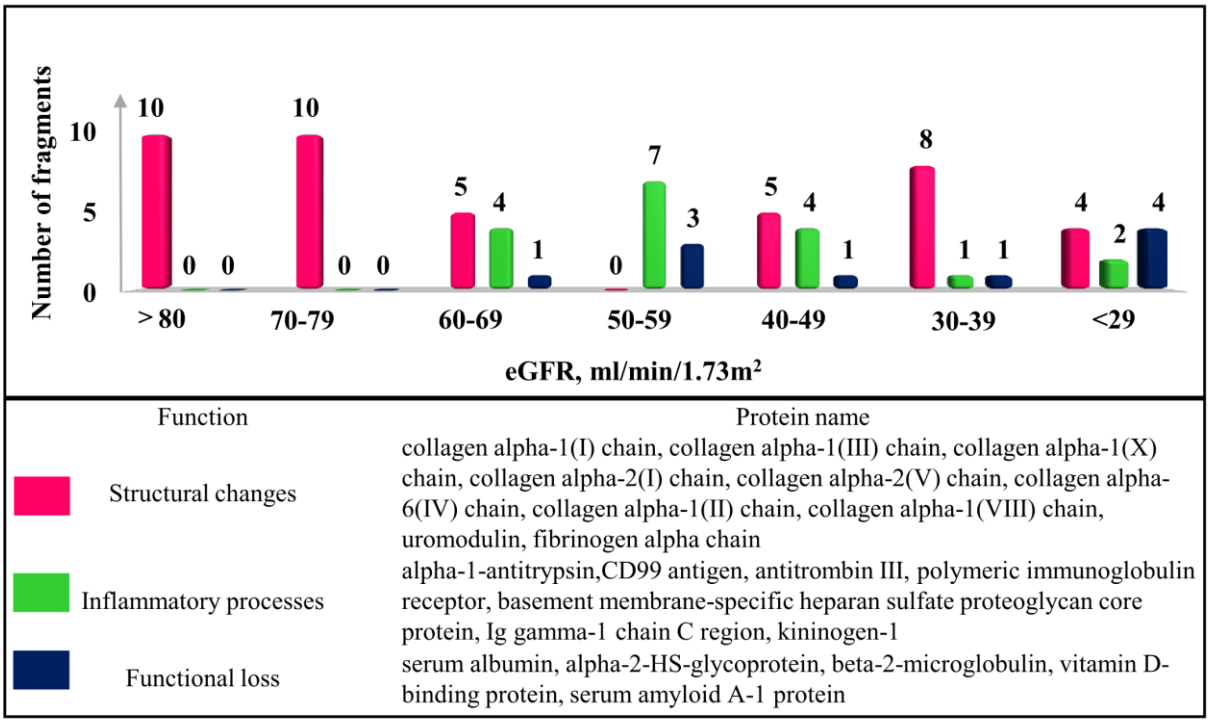

Figure 2. Number and protein origin of most significant urinary peptides in each eGFR stratum. Peptides of structural changes are in fuchsia; peptides of inflammatory processes are coloured in green; peptides indicate functional loss are in blue.

3.2 Publication 2: Smith A, L'Imperio V, De Sio G, Ferrario F, Scalia C, Dell'Antonio G, Pieruzzi F, Pontillo C, Filip S, Markoska K, Granata A, Spasovski G, Jankowski J, Capasso G, Pagni F, Magni F. α-1-Antitrypsin detected by MALDI imaging in the study of glomerulonephritis: Its relevance in chronic kidney disease progression, Proteomics. 2016 16(11-12):1759-66.

The study was focused on the investigation of fresh-frozen renal biopsies taken from patients with a histological diagnosis affected by glomerulonephritis (GN) of focal segmental glomerulosclerosis (FSGS) (n=6), IgA nephropathy (IgAN) (n=6), and membranous glomerulonephritis (MGN) (n=7). MALDI-MSI profiles of the aforementioned patients were compared to normal cortical biopsies of patients with no history of renal diseases (controls, n=4). The comparison of MALDI-MSI between pool of samples from all patients with all the three glomerulonephritis (GNs) and control group showed three signals at m/z 4,025, 4,085 and 4,963 which displayed a significant higher intensity (AUC>0.8, P<0.05) compared to controls. Due to the fact that several sub-diagnosis of renal disease were used, as next step specific protein profiles from FSGS, IgAN and MGN were compared each other. Signals at m/z 4,025, 4,085 were significantly higher in FSGS patients rather than in patients affected by IgAN and MGN (AUC=0.84 and 0.82, respectively). On the other hand, mass spectrum of IgAN patients highlighted the presence of two signals at m/z 4,964 and 5,072 with higher intensity compared to spectrum obtained from FSGS patients. When comparing spectrum of IgAN to MGN patients, two signals (at m/z 5,072 and 6,180) were reported with higher intensity in IgAN patients. As next step, the proteomic findings were also correlated to the histological images, highlighting that IgAN spectrum (in common with the spectra of FSGS patients) were related to a group of three sections of sclerotic glomeruli. Inspection of virtual microdissections of those specific glomeruli, showed a similar distribution between IgAN and FSGS patients. In general, microdissections of glomeruli confirmed similarity of IgAN and FSGS that were observed in protein profiles analysed by MALDI-MSI. Peptide signals at m/z 4,025 and 4,085 were able to distinguish sclerotic glomeruli among the three CKD etiologies. Therefore, these particular signals were sequenced via MALDI-TOF/TOF. The signal at m/z 4,025 was identified as tryptic peptide of α1-antitrypsin (A1AT) protein having the following sequence: IPPEVKFNKPFVFLMIEQ NTKSPLFMGKVVNPTQK. Immunohistochemistry of A1AT on renal biopsies confirmed the positive presence of this protein in sclerotic regions of IgAN and FSGS. Moreover, staining of A1AT showed infiltration of A1AT in the cytoplasm of podocytes, while the podocytes of mesangioproliferative renal biopsies pattern of IgAN and normal glomeruli displayed negative staining for A1AT. These results are shown in **Figure 3**. Of note, fragments of A1AT are also present in the proteome-based "CKD273"-classifier. Five of these fragments partially overlapped with the A1AT fragment identified in tissue samples. In total 18 fragments of A1AT are present in the proteome-based classifier, showing an up-regulation of this protein in patients with CKD compared to control group (absence of renal disease). To further correlate tissue findings with peptidomic data, urinary A1AT fragments were also found to be correlated with CKD progression in Schanstra et al [8]. Among them, four

fragments partially overlapped with the A1AT fragment identified in study. Moreover, identical tryptic fragment of A1AT was also identified in a different sample cohort with increased abundance in the urine of patients classified as CKD progressors compared to non-progressors (ratio progressors/non-progressors = 5.3, p = 0.02).

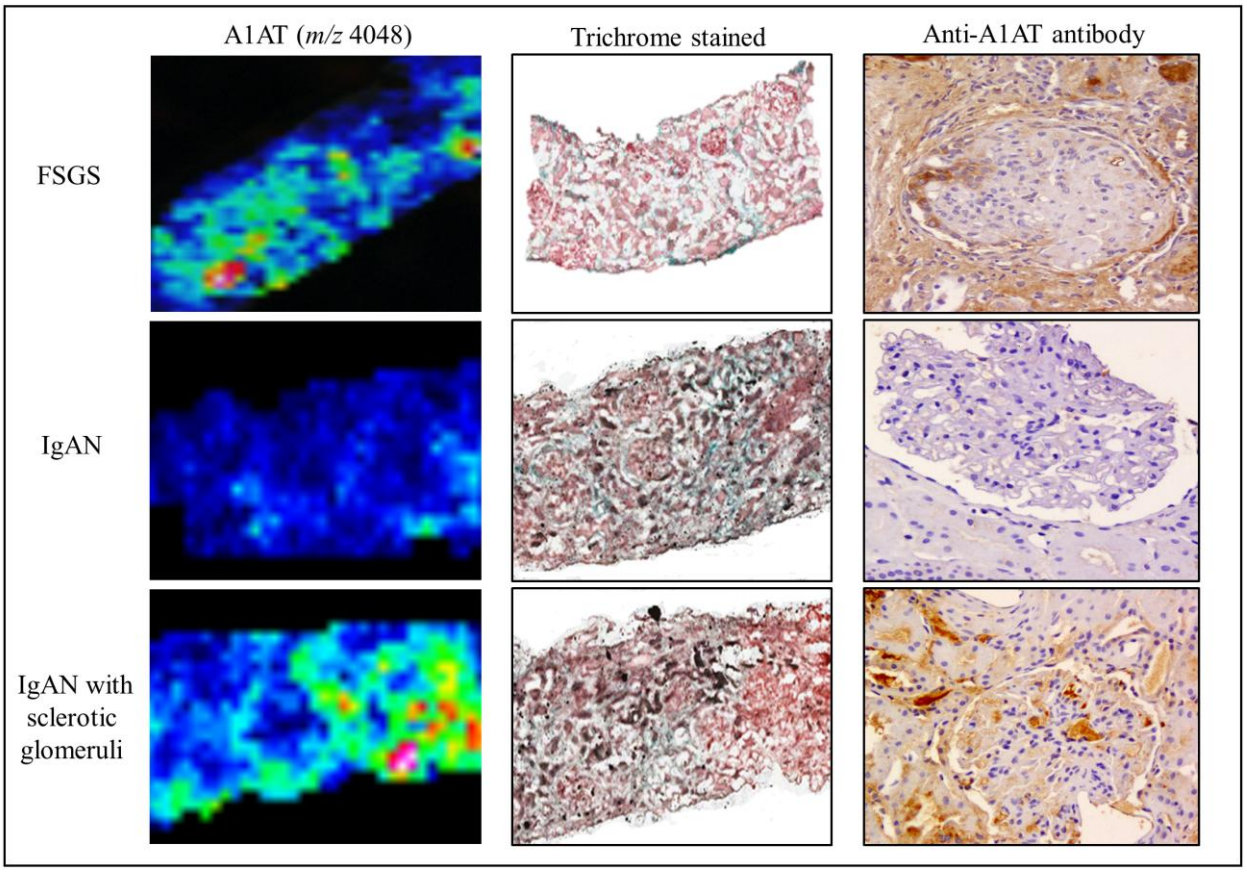

Figure 3. α-1-antitrypsin identified via MALDI-MSI and CE-MS for glomerulonephritis and diagnosis of CKD, respectively. (Left) The molecular distribution of m/z 4,048 (A1AT) in sections of bioptic renal tissue, obtained using MALDI-MSI, (center) trichrome-stained image of the identical sections used for MALDI-MS imaging and (right) immunohistochemical staining with the anti- A1AT antibody from a validation set of patients. The collection of images is derived from patients with FSGS (top), IgAN (middle), and IgAN with sclerotic glomeruli (bottom).

3.3 Publication 3: Siwy J, Zürbig P, Argiles A, Beige J, Haubitz M, Jankowski J, Julian B A, Linde P G, Marx D, Mischak H, Mullen W, Novak J, Ortiz A, Persson F, Pontillo C, Rossing P, Rupprecht H, Schanstra J P, Vlahou A, Vanholder R, Non-invasive diagnosis of chronic kidney diseases using urinary proteome analysis Nephrology Dialysis Transplantation, 2016 doi: 10.1093/ndt/gfw337

In the current study, we explored the potential of urinary peptides analysed via CE-MS to define different types of CKDs for non-invasive diagnosis as possible alternative to renal biopsies. The cohort included 1,180 datasets [19] of subjects displaying eight etiologies of CKD, such as focal segmental glomerulosclerosis (FSGS), IgA nephropathy (IgAN), minimal-change disease (MCD), membranous nephropathy (MN), diabetic nephropathy (DN), hypertensive nephrosclerosis (N), lupus nephritis (LN) and vasculitis-induced kidney disease (vasculitis). Number of patients for each group are reported in the material and methods section. However, datasets from N and DN were pooled together as certain evidence of the presence of these diseases was not confirmed by renal biopsies. Using the non-parametric Wilcoxon test, 287 urinary peptides disease-specific were defined for FSGS, 291 for MCD, 311 for MN, 172 for LN, 509 for vasculitis-induced kidney disease 116 for IgAN and 619 for DN&N. Those specific biomarkers were assembled into seven different peptide-based classifiers targeted for each specific CKD etiology. The AUCs of the ROC-analyses of all classifiers ranged from 0.77 to 0.95 applying the aforementioned classifiers into an independent cohort composed by 474 subjects (in which each CKD etiology was compared with all the other types of CKD).

Figure 4 reported ROC-curves of all the different CKD etiologies. 488 peptide-sequences were obtained via CE- and LC-MS/MS, which represent 42% of the total amount of the defined urinary biomarkers. Out of 488 biomarkers; 328 were significantly increased or decreased with only one CKD etiology. Next, we aimed to investigate the abundance and fold-changes of the individual peptides among the different CKDs. For this purpose, only peptides with a fold-change of at least 2 were accepted. Moreover, to allow comparison among the different CKD groups, peptides displaying the same regulation (increasing or decreasing) were combined together. In the case of DN&N, 5 different hemoglobin peptide fragments were significantly decreased in the DN&N group in comparison to other CKD etiologies. Down-regulation of small proline-rich protein 3 and leucine-rich repeat-containing protein 25 and up-regulation of clusterin and apolipoprotein fragments (both with the same p-value) were observed. For FSGS, we reported up-regulation of collagen fragments and down-regulation of fibrinogen, polymeric immunoglobulin receptor and Golgi-associated olfactory signaling regulator. For IgAN up-regulation of hemoglobin (in contrast

to the DN&N group), leucin-rich repeat-containing protein 25, small proline-rich protein fragments and sodium/potassium transporting ATPase were increased. For the LN cohort, up-regulated collagen, uromodulin, and protein S100-A9 fragments and down-regulated of clusterin fragments were reported. In the MCD group, we observed up-regulated fragments of collagen, uromodulin and apolipoprotein C-IV. However, collagen fragments and two fragments of beta-2-microglobulin were also reported as down-regulated. In the MN group, we observed up-regulated fragments of cystatin-A, biorientation of chromosomes in cell division protein 1, uromodulin and plasminogen. In the vasculitis group, the lowest p-values were observed for the up-regulated hemoglobin, sodium/potassium-transporter ATPase, collagen and small proline-rich protein 3.

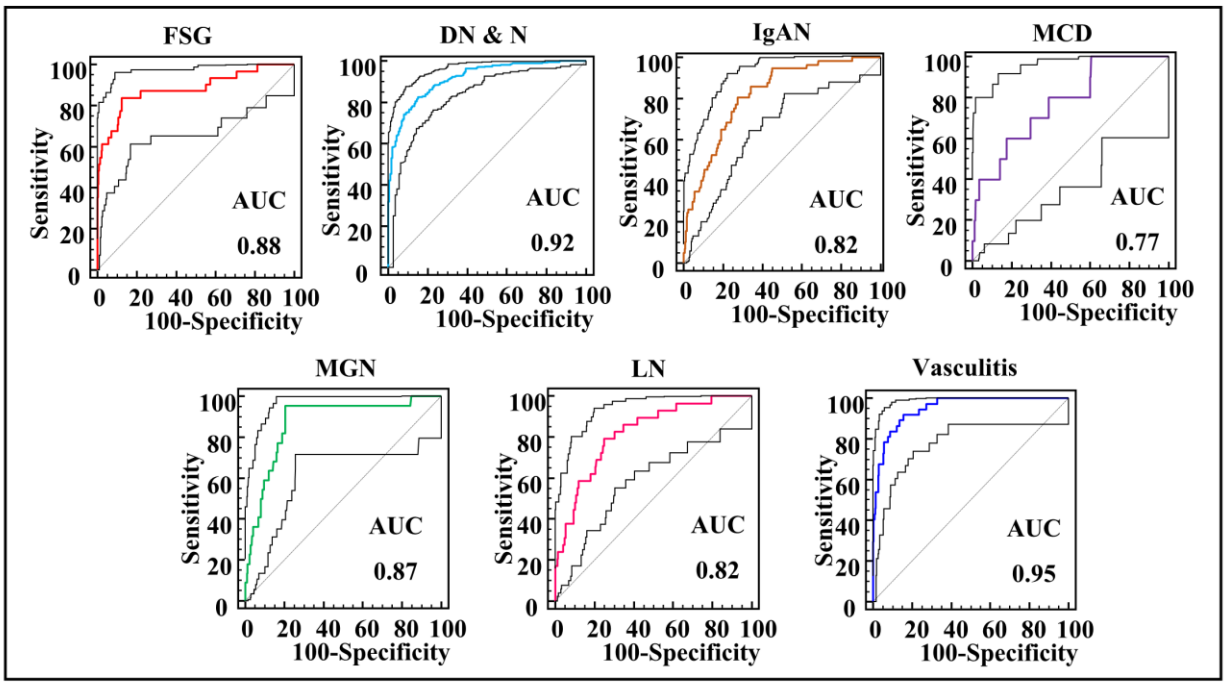

Figure 4. ROC-analysis of the classification results for each developed classifier applied to independent samples from the validation set, for each type of CKD. 95% interval of confidence is reported in black colour per each type of CKD. In the figure: DN-diabetic nephropathy; FSG-focal segmental glomerulosclerosis; GN-glomerulonephritis; IgAN-IgA nephropathy; LN-lupus; MCD-minimal-change disease; MGN-membranous glomerulonephritis; MN-membranous nephropathy; N-nephrosclerosis; Vasculitis-vasculitis-induced kidney disease.

4. Discussion

The possibility of non-invasive diagnosis and prognosis of chronic kidney diseases was assessed in this thesis, investigating peptidome profiles of CKD patients. As first aim, it was explored the ability of using a previous established peptide-based classifier ("CKD273"-classifier) [6] for prognosis of CKDs. In this study, a new approach for defining early progression of CKD was established exploring urinary "CKD273"-classifier [6] and *de novo* urinary peptides associated with different eGFR stratum. A recent study has shown the relevance of early intervention in CKD (diabetic nephropathy) using RAS inhibitors, which is more beneficial in delaying end-stage renal disease than intervention at later stages [27]. Therefore, optimization of biomarkers towards specific eGFR stratum and in early CKD stages was studied. The results showed that while at early CKD stages the "CKD273"-classifier outperformed albuminuria in the detection of fast progressors (eGFR decline >5ml/min/1.73m^2 per year) [11], albuminuria performed better in CKD patients with late stage disease. Interestingly, in the eGFR-groups between <80 and 40-49 ml/min/1.73m^2 the scores of the "CKD273"-classifier were constant irrespective of patients being cases or control, while albuminuria displayed increasing values in this eGFR baseline range, but starting from a low value of baseline eGFR (<50 ml/min/1.73m^2). The reason why the performance of the proteome classifier is more constant in the classification of patients might be related to the fact that 273 different peptides are combined together and they are able to better depict the complexity of a heterogeneous disease, such as CKD. However, due to the fact that performance of both albuminuria and "CKD273"-classifier in certain eGFR stratum were not optimal, we have investigated the top ten abundant individual urinary associated with single eGFR groups (≥80, 79-70, 69-60, 59-50, 49-40, 39-30, <29 mL/min/1.73m^2).

As showed in the **Publication 1**, we were able to potentially link individual urinary peptides to the patho-physiology of CKD progression. At early CKD stage (eGFR >80-70 ml/min/1.73m^2), several collagen peptides were present suggesting changes in extracellular matrix (ECM) turnover. Collagen type I and III are known to be deposited in early stages of renal fibrosis [28] [29]. During fibrosis, the formation of scar tissue in the interstitial space is the result of the excessive accumulation of ECM components, which is connected to alterations of metalloproteases (MMPs) and their inhibitors responsible for maintaining the equilibrium between formation and degradation of ECM proteins [30]. The excretion of collagen peptides in urine of samples with early CKD stage might explain the good performance of the "CKD273"-classifier at this stages. Indeed, "CKD273"-classifier is composed of 202 out 273 collagen fragments and acts as biomarker of ECM remodeling, identifying molecular processes occurring in the early phases

of fibrogenesis. Another protein found to be associated with early disease stages was uromodulin. Literature searching suggested that excretion of uromodulin indicates early medullary dysfunction in diabetes mellitus type I [31] and is associated in the development of ECM by early leakage from the tubular lumen to the tubular intersitium, inducing inflammation and fibrosis [32]. At moderately advanced disease (eGFR:69-40ml/min/1.73m^2), peptides associated with inflammatory process were highlighted, such as alpha-1-antitrypsin (A1AT). This protein was found to be highly present in acute kidney injury [33]. In our study, we suggested an alternative function of A1AT in urine at moderate advanced disease stages as mediator of inflammatory processes in the kidney, which can induce progression of disease to more severe CKD stages. At late stage disease (eGFR <39 ml/min/1.73m^2) the glomerular damage results in the loss of filtration barrier with the appearance of blood proteins like serum albumin, vitamin D-binding protein, transthyretin, serum amyloid A-1 protein, and alpha-1-antitrypsin in the urine. However, at this late stage damage to the glomeruli in the kidney may be irreversible and therapy could not be curative.

Despite the fact that the clinical use of those individual urinary peptides as new potential biomarkers still needs to be validated in additional studies, we could have evidence of the presence of A1AT fragments in renal fresh frozen biopsies of patients with focal segmental glomerulosclerosis (FSGS), IgA nephropathy (IgAN) and membranous glomerulonephritis (MGN), as shown in **Publication 2**. Using MALDI-MSI the signal at m/z 4,048 was identified as a peptide fragment of A1AT (IPPEVKFNKPFVFLMIEQ NTKSPLFMGKVVNPTQK). The immunohistochemistry staining against this protein demonstrated localization of A1AT in the cytoplasm of podocytes present in sclerotic glomeruli. This protein could be related with "podocyte stress theory"[34] [35] [36]. Smaller fragments of the peptide derived from the A1AT protein that we have identified in **Publication 2** were reported in urine samples of patients with glomerulonephritis [6]. In particular, several fragments of A1AT are already reported in the "CKD273"-classifier [6] used in **Publication 1**. 18 different fragments of this protein are present in the "CKD273"-classifier. All of them were upregulated in CKD patients in comparison to the control groups. Five of these fragments partially overlapped with the A1AT fragment we identified in tissue samples. Moreover, urinary A1AT fragments were also found to be correlated with CKD progression in Schanstra et al. [8]. Among them, four fragments partially overlapped with the A1AT fragment identified in tissue. The combined findings from tissue MALDI-MSI and urinary peptidomics related to A1AT suggested relation between the presence of glomerulosclerosis and the aforementioned podocyte changes with early signs of CKD disease progression [37]. However, in order to correlate tissue findings with urinary peptidomics results, variability derived from the

use of different techniques (CE-MS and MALDI-MSI) should be carefully and further interpreted. Moreover, the consistency between tissue analysis and urinary results should be further investigated by studying both biopsies from renal tissue and urinary profiles from same patients within an appropriate cohort to investigate specific markers of several CKD aetiologies.

The possibility of diagnosis of different CKD etiologies by the analysis of urine samples via CE-MS, was also investigated as reported in **Publication 3**. Potential urinary biomarkers associated with focal segmental glomerulosclerosis (FSGS), IgA nephropathy (IgAN), minimal-change disease (MCD), membranous nephropathy (MN), diabetic nephropathy (DN), hypertensive nephrosclerosis (N), lupus nephritis (LN) and vasculitis-induced kidney disease (vasculitis) were highlighted. We decided to assess these diseases by the analysis of urinary peptides because their diagnosis, generally based on proteinuria and other micro-vascular complications, may not always be correct [38]. Therefore, our scope was to differentiate these several categories of renal diseases that currently are diagnosed by biopsy and that require specific therapeutic interventions. Although the classifiers defined in the **Publication 3** were composed of multiple peptides specific for a single CKD etiology, certain similarities in the individual biomarkers between some CKD etiologies could be observed. In general, significant changes in abundance of many collagen fragments were observed in almost all investigated CKD diseases. Clusterin fragments were reported up-regulated in patients with DN and N, comparing with other CKD etiologies. This results is confirmed in the literature by Chu et al. that showed urinary clusterin is able to discriminate DN from healthy controls [39]. In FSGS, IgAN, LN and MCD groups all clusterin fragments were down-regulated. Our findings are supported by many studies that studied MN, FSGS children with nephrotic syndrome and systemic lupus erythematosus (SLE), in which serum and urinary clusterin were reported to be down-regulated [40;41]. Hemoglobin fragments were down regulated in DN and N patients but up-regulated in IgAN and vasculitis groups, as expected in patients with haematuria. In our study, we reported high similarity of urinary peptides excretion between the IgAN and ANCA-associated groups in comparison to other CKD etiologies. This can be explained by the fact that both diseases display usually a prominent glomerular inflammation [42] [43]. MCD and FSGS groups shared a decreased abundance of fibrinogen peptides. MCD and FSGS are primary podocytopathies and thus may display similarity in the pathophiology that lead to severe injury with loss of podocytes in FSGS. LN group was characterized by the S100-A9 fragment, which was the most specific peptides compared to other CKD etiologies. This is a calcium- and zinc-binding protein that is involved in the regulation of inflammatory processes and immune response. A significantly higher level of this protein together with protein S100A8 in

serum samples of patients with systemic lupus erythematosus compared to healthy controls was already observed by Soyfoo et al [44].

In conclusions, urine and renal bioptic samples were analysed for the identification of putative biomarkers relative to CKD progression and different CKD etiologies. Each of these studies allows a quite comprehensive information on the pathology of CKD. Based on the result of this thesis, it is expected that this approach will be helpful for management of patients at risk of advanced renal failure by allowing a more targeted early intervention. Moreover, the use of clinical proteomics for non-invasive diagnosis of several CKD etiologies can be of guidance for disease evaluation and/or treatment, when validated in further studies including a larger number of patients.

5. Bibliography

(1) Schulz-Knappe P, Zucht HD, Heine G, Jurgens M, Hess R, Schrader M: Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. Comb Chem High Throughput Screen 2001;4:207-217.

(2) Jankowski J, Schanstra JP, Mischak H: Body fluid peptide and protein signatures in diabetic kidney diseases. Nephrol Dial Transplant 2015; 30 Suppl 4:iv43-iv53.

(3) Salem S, Jankowski V, Asare Y, Liehn E, Welker P, Raya-Bermudez A, Pineda-Martos C, Rodriguez M, Munoz-Castaneda JR, Bruck H, Marx N, Machado FB, Staudt M, Heinze G, Zidek W, Jankowski J: Identification of the Vasoconstriction-Inhibiting Factor (VIF), a Potent Endogenous Cofactor of Angiotensin II Acting on the Angiotensin II Type 2 Receptor. Circulation 2015;131:1426-1434.

(4) Mischak H, Schanstra JP: CE-MS in biomarker discovery, validation, and clinical application. Proteomics Clin Appl 2011;5:9-23.

(5) Mainini V, Pagni F, Ferrario F, Pieruzzi F, Grasso M, Stella A, Cattoretti G, Magni F: MALDI imaging mass spectrometry in glomerulonephritis: feasibility study. Histopathology 2014;64:901-906.

(6) Good DM, Zurbig P, Argiles A, Bauer HW, Behrens G, Coon JJ, Dakna M, Decramer S, Delles C, Dominiczak AF, Ehrich JH, Eitner F, Fliser D, Frommberger M, Ganser A, Girolami MA, Golovko I, Gwinner W, Haubitz M, Herget-Rosenthal S, Jankowski J, Jahn H, Jerums G, Julian BA, Kellmann M, Kliem V, Kolch W, Krolewski AS, Luppi M, Massy Z, Melter M, Neususs C, Novak J, Peter K, Rossing K, Rupprecht H, Schanstra JP, Schiffer E, Stolzenburg JU, Tarnow L, Theodorescu D, Thongboonkerd V, Vanholder R, Weissinger EM, Mischak H, Schmitt-Kopplin P: Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol Cell Proteomics 2010;9:2424-2437.

(7) Roscioni SS, de ZD, Hellemons ME, Mischak H, Zurbig P, Bakker SJ, Gansevoort RT, Reinhard H, Persson F, Lajer M, Rossing P, Lambers Heerspink HJ: A urinary peptide biomarker set predicts worsening of albuminuria in type 2 diabetes mellitus. Diabetologia 2013;56:259-267.

(8) Schanstra JP, Zurbig P, Alkhalaf A, Argiles A, Bakker SJ, Beige J, Bilo HJ, Chatzikyrkou C, Dakna M, Dawson J, Delles C, Haller H, Haubitz M, Husi H, Jankowski J, Jerums G, Kleefstra N, Kuznetsova T, Maahs DM, Menne J, Mullen W, Ortiz A, Persson F, Rossing P, Ruggenenti P, Rychlik I, Serra AL, Siwy J, Snell-Bergeon J, Spasovski G, Staessen JA, Vlahou A, Mischak H, Vanholder R: Diagnosis and Prediction of CKD Progression by Assessment of Urinary Peptides. J Am Soc Nephrol 2015;26:999-2010.

(9) Siwy J, Schanstra JP, Argiles A, Bakker SJ, Beige J, Boucek P, Brand K, Delles C, Duranton F, Fernandez-Fernandez B, Jankowski ML, Al KM, Kunt T, Lajer M, Lichtinghagen R, Lindhardt M, Maahs DM, Mischak H, Mullen W, Navis G, Noutsou M, Ortiz A, Persson F, Petrie JR, Roob JM, Rossing P, Ruggenenti P, Rychlik I, Serra AL, Snell-Bergeon J, Spasovski G, Stojceva-Taneva O, Trillini M, von der LH, Winklhofer-Roob BM, Zurbig P, Jankowski J: Multicentre prospective validation of a urinary peptidome-based classifier for the diagnosis of type 2 diabetic nephropathy. Nephrol Dial Transplant 2014;29:1563-1570.

(10) Zurbig P, Jerums G, Hovind P, MacIsaac RJ, Mischak H, Nielsen SE, Panagiotopoulos S, Persson F, Rossing P: Urinary proteomics for early diagnosis in diabetic nephropathy. Diabetes 2012;61:3304-3313.

(11) KDOQI Clinical Practice Guideline for Diabetes and CKD: 2012 Update: Am J Kidney Dis 2012;60:850-886.

(12) Coresh J, Turin TC, Matsushita K, Sang Y, Ballew SH, Appel LJ, Arima H, Chadban SJ, Cirillo M, Djurdjev O, Green JA, Heine GH, Inker LA, Irie F, Ishani A, Ix JH, Kovesdy CP, Marks A, Ohkubo T, Shalev V, Shankar A, Wen CP, de Jong PE, Iseki K, Stengel B, Gansevoort RT, Levey AS: Decline in estimated glomerular filtration rate and subsequent risk of end-stage renal disease and mortality. JAMA 2014;311:2518-2531.

(13) Parving HH, Persson F, Rossing P: Microalbuminuria: a parameter that has changed diabetes care. Diabetes Res Clin Pract 2015;107:1-8.

(14) Mischak H, Delles C, Vlahou A, Vanholder R: Proteomic biomarkers in kidney disease: issues in development and implementation. Nat Rev Nephrol 2015.

(15) Miller WG, Bruns DE, Hortin GL, Sandberg S, Aakre KM, McQueen MJ, Itoh Y, Lieske JC, Seccombe DW, Jones G, Bunk DM, Curhan GC, Narva AS: Current issues in measurement and reporting of urinary albumin excretion. Clin Chem 2009;55:24-38.

(16) Perkins BA, Ficociello LH, Roshan B, Warram JH, Krolewski AS: In patients with type 1 diabetes and new-onset microalbuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. Kidney Int 2010;77:57-64.

(17) Costacou T, Ellis D, Fried L, Orchard TJ: Sequence of progression of albuminuria and decreased GFR in persons with type 1 diabetes: a cohort study. Am J Kidney Dis 2007;50:721-732.

(18) Brenner BM, Cooper ME, de ZD, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med 2001;345:861-869.

(19) Siwy J, Mullen W, Golovko I, Franke J, Zurbig P: Human urinary peptide database for multiple disease biomarker discovery. Proteomics Clin Appl 2011;5:367-374.

(20) Stalmach A, Albalat A, Mullen W, Mischak H: Recent advances in capillary electrophoresis coupled to mass spectrometry for clinical proteomic applications. Electrophoresis 2013;34:1452-1464.

(21) Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, III, Feldman HI, Kusek JW, Eggers P, Van LF, Greene T, Coresh J: A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150:604-612.

(22) Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24:959-977.

(23) Neuhoff N, Kaiser T, Wittke S, Krebs R, Pitt A, Burchard A, Sundmacher A, Schlegelberger B, Kolch W, Mischak H: Mass spectrometry for the detection of differentially expressed proteins: a comparison of surface-enhanced laser desorption/ionization and capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 2004;18:149-156.

(24) Jantos-Siwy J, Schiffer E, Brand K, Schumann G, Rossing K, Delles C, Mischak H, Metzger J: Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. J Proteome Res 2009;8:268-281.

(25) Zurbig P, Renfrow MB, Schiffer E, Novak J, Walden M, Wittke S, Just I, Pelzing M, Neususs C, Theodorescu D, Root KE, Ross MM, Mischak H: Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. Electrophoresis 2006;27:2111-2125.

(26) Hochberg Y, Benjamini Y: More powerful procedures for multiple significance testing. Stat Med 1990;9:811-818.

(27) Schievink B, Kropelin T, Mulder S, Parving HH, Remuzzi G, Dwyer J, Vemer P, de ZD, Lambers Heerspink HJ: Early renin-angiotensin system intervention is more beneficial than late intervention in delaying end-stage renal disease in patients with type 2 diabetes. Diabetes Obes Metab 2016;18:64-71.

(28) Johnson TS, Haylor JL, Thomas GL, Fisher M, El Nahas AM: Matrix metalloproteinases and their inhibitions in experimental renal scarring. Exp Nephrol 2002;10:182-195.

(29) Chakraborty J, Below AA, Solaiman D: Tamm-Horsfall protein in patients with kidney damage and diabetes. Urol Res 2004;32:79-83.

(30) Genovese F, Manresa AA, Leeming DJ, Karsdal MA, Boor P: The extracellular matrix in the kidney: a source of novel non-invasive biomarkers of kidney fibrosis? Fibrogenesis Tissue Repair 2014;7:4.

(31) Zimmerhackl LB, Pfleiderer S, Kinne R, Manz F, Schuler G, Brandis M: Tamm-Horsfall-Protein excretion as a marker of ascending limb transport indicates early renal tubular damage in diabetes mellitus type I. J Diabet Complications 1991;5:112-114.

(32) Eddy AA: Scraping fibrosis: UMODulating renal fibrosis. Nat Med 2011;17:553-555.

(33) Zager RA, Johnson AC, Frostad KB: Rapid renal alpha-1 antitrypsin gene induction in experimental and clinical acute kidney injury. PLoS One 2014;9:e98380.

(34) Smeets B, Moeller MJ: Parietal epithelial cells and podocytes in glomerular diseases. Semin Nephrol 2012;32:357-367.

(35) Moeller MJ, Smeets B: Role of parietal epithelial cells in kidney injury: the case of rapidly progressing glomerulonephritis and focal and segmental glomerulosclerosis. Nephron Exp Nephrol 2014;126:97.

(36) Kretzler M: Role of podocytes in focal sclerosis: defining the point of no return. J Am Soc Nephrol 2005;16:2830-2832.

(37) Kriz W, Lemley KV: A potential role for mechanical forces in the detachment of podocytes and the progression of CKD. J Am Soc Nephrol 2015;26:258-269.

(38) Fiorentino M, Bolignano D, Tesar V, Pisano A, Van BW, D'Arrigo G, Tripepi G, Gesualdo L: Renal Biopsy in 2. Am J Nephrol 2016;43:1-19.

(39) Chu L, Fu G, Meng Q, Zhou H, Zhang M: Identification of urinary biomarkers for type 2 diabetes using bead-based proteomic approach. Diabetes Res Clin Pract 2013;101:187-193.

(40) Ghiggeri GM, Bruschi M, Candiano G, Rastaldi MP, Scolari F, Passerini P, Musante L, Pertica N, Caridi G, Ferrario F, Perfumo F, Ponticelli C: Depletion of clusterin in renal diseases causing nephrotic syndrome. Kidney Int 2002;62:2184-2194.

(41) Newkirk MM, Apostolakos P, Neville C, Fortin PR: Systemic lupus erythematosus, a disease associated with low levels of clusterin/apoJ, an antiinflammatory protein. J Rheumatol 1999;26:597-603.

(42) Haubitz M, Good DM, Woywodt A, Haller H, Rupprecht H, Theodorescu D, Dakna M, Coon JJ, Mischak H: Identification and validation of urinary biomarkers for differential diagnosis and evaluation of therapeutic intervention in anti-neutrophil cytoplasmic antibody-associated vasculitis. Mol Cell Proteomics 2009;8:2296-2307.

(43) Moreno JA, Martin-Cleary C, Gutierrez E, Toldos O, Blanco-Colio LM, Praga M, Ortiz A, Egido J: AKI associated with macroscopic glomerular hematuria: clinical and pathophysiologic consequences. Clin J Am Soc Nephrol 2012;7:175-184.

(44) Soyfoo MS, Roth J, Vogl T, Pochet R, Decaux G: Phagocyte-specific S100A8/A9 protein levels during disease exacerbations and infections in systemic lupus erythematosus. J Rheumatol 2009;36:2190-2194.

6. Appendix

6.1 Affidavit

I, **Claudia Pontillo,** certify under penalty of perjury by my own signature that I have submitted the thesis on the topic **Clinical peptidomics for non-invasive diagnosis and prognosis of 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 section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

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 Detailed Declaration of Contribution

Claudia Pontillo had the following share in the following publication:

Publication 1: Pontillo C, Lotte Jacobs, Staessen JA, Schanstra JP, Rossing P, Heerspink HJL, Siwy J, Mullen W, Vlahou A, Mischak H, Vanholder R, Zürbig P, Jankowski J A urinary proteome-based classifier for the early detection of decline in glomerular filtration, Nephrology Dialysis Transplantation, 2016
Contribution in detail: data selection, data processing, data analysis, statistical analysis and writing of the manuscript. (IF: 3.57)

Publication 2: Smith A, L'Imperio V, De Sio G, Ferrario F, Scalia C, Dell'Antonio G, Pieruzzi F, Pontillo C, Filip S, Markoska K, Granata A, Spasovski G, Jankowski J, Capasso G, Pagni F, Magni F alpha-1-antitrypsin detected by MALDI-Imaging in the study of glomerulonephritis: its relevance in chronic kidney disease progression, Proteomics, 2016
Contribution in detail: integration of MALDI findings (data) with urinary peptidomics data, critical evaluation of the manuscript and acceptance of the final version. (IF: 1.56)

Publication 3: Siwy J, Zürbig P, Argiles A, Beige J, Haubitz M, Jankowski J, Julian B A, Linde P G, Marx D, Mischak H, Mullen W, Novak J, Ortiz A, Persson F, Pontillo C, Rossing P, Rupprecht H, Schanstra J P, Vlahou A, Vanholder R
Non-invasive diagnosis of chronic kidney diseases using urinary proteome analysis, Nephrology Dialysis Transplantation, 2016 (IF: 3.57)

Contribution in detail: clinical data collection, critical evaluation of the manuscript and acceptance of the final version.

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

6.3 Selected publications

Publication 1: Pontillo C, Lotte Jacobs, Staessen JA, Schanstra JP, Rossing P, Heerspink HJL, Siwy J, Mullen W, Vlahou A, Mischak H, Vanholder R, Zürbig P, Jankowski J A urinary proteome-based classifier for the early detection of decline in glomerular filtration, Nephrology Dialysis Transplantation, 2016 Excluded article. Full text link, DOI: http://dx.doi.org/10.1093/ndt/gfw239

Publication 2: Smith A, L'Imperio V, De Sio G, Ferrario F, Scalia C, Dell'Antonio G, Pieruzzi F, Pontillo C, Filip S, Markoska K, Granata A, Spasovski G, Jankowski J, Capasso G, Pagni F, Magni F alpha-1-antitrypsin detected by MALDI-Imaging in the study of glomerulonephritis: its relevance in chronic kidney disease progression, Proteomics, 2016

Publication 3: Siwy J, Zürbig P, Argiles A, Beige J, Haubitz M, Jankowski J, Julian B A, Linde P G, Marx D, Mischak H, Mullen W, Novak J, Ortiz A, Persson F, Pontillo C, Rossing P, Rupprecht H, Schanstra J P, Vlahou A, Vanholder R Non-invasive diagnosis of chronic kidney diseases using urinary proteome analysis, Nephrology Dialysis Transplantation, 2016 Excluded article. Full text link, DOI: http://dx.doi.org/10.1093/ndt/gfw337

RESEARCH ARTICLE

α-1-Antitrypsin detected by MALDI imaging in the study of glomerulonephritis: Its relevance in chronic kidney disease progression

Andrew Smith[1], Vincenzo L'Imperio[2*], Gabriele De Sio[1], Franco Ferrario[2], Carla Scalia[2], Giacomo Dell'Antonio[3], Federico Pieruzzi[4], Claudia Pontillo[5,6], Szymon Filip[6,7], Katerina Markoska[8], Antonio Granata[9], Goce Spasovski[8], Joachim Jankowski[10], Giovambattista Capasso[11], Fabio Pagni[2] and Fulvio Magni[1]*

[1] Unit of Proteomics, Department of Health Sciences, University of Milano-Bicocca, Monza, Italy
[2] Unit of Pathology, Department of Surgery and Translational Medicine, University of Milano-Bicocca, Monza, Italy
[3] Department of Pathology, San Raffaele Hospital, Milan, Italy
[4] Unit of Nephrology, Department of Health Sciences, University of Milano-Bicocca, Milan, Italy
[5] Mosaiques Diagnostics GmbH, Hannover, Germany
[6] Charité – Universitätsmedizin Berlin, Berlin, Germany
[7] Biomedical Research Foundation Academy of Athens, Athens, Greece
[8] Department of Nephrology, Medical Faculty, University of Skopje, Skopje, Macedonia
[9] Department of Nephrology, Ospedale San Giovanni di Dio, Agrigento, Italy
[10] Institute for Molecular Cardiovascular Research, University Hospital RWTH Aachen, Aachen, Germany
[11] Department of Cardio-Thoracic and Respiratory Science, Second University of Naples, Naples, Italy

Received: October 22, 2015
Revised: November 16, 2015
Accepted: January 4, 2016

Idiopathic glomerulonephritis (GN), such as membranous glomerulonephritis, focal segmental glomerulosclerosis (FSGS), and IgA nephropathy (IgAN), represent the most frequent primary glomerular kidney diseases (GKDs) worldwide. Although the renal biopsy currently remains the gold standard for the routine diagnosis of idiopathic GN, the invasiveness and diagnostic difficulty related with this procedure highlight the strong need for new diagnostic and prognostic biomarkers to be translated into less invasive diagnostic tools. MALDI-MS imaging MALDI-MSI was applied to fresh-frozen bioptic renal tissue from patients with a histological diagnosis of FSGS ($n = 6$), IgAN, ($n = 6$) and membranous glomerulonephritis ($n = 7$), and from controls ($n = 4$) in order to detect specific molecular signatures of primary glomerulonephritis. MALDI-MSI was able to generate molecular signatures capable to distinguish between normal kidney and pathological GN, with specific signals (m/z 4025, 4048, and 4963) representing potential indicators of chronic kidney disease development. Moreover, specific disease-related signatures (m/z 4025 and 4048 for FSGS, m/z 4963 and 5072 for IgAN) were detected. Of these signals, m/z 4048 was identified as α-1-antitrypsin and was shown to be localized to the podocytes within sclerotic glomeruli by immunohistochemistry. α-1-Antitrypsin could be one of the markers of podocyte stress that is correlated with the development of FSGS due to both an excessive loss and a hypertrophy of podocytes.

Keywords:
Biomedicine / Biopsy / Glomerulonephritis / MALDI imaging / Mass spectrometry

Correspondence: Dr. Fulvio Magni, Department of Health Sciences, University of Milano-Bicocca, Piazza dell'Ateneo Nuovo, 1, 20126 Milano, Italy
E-mail: fulvio.magni@unimib.it
Fax: +39-02-6448252

Abbreviations: **A1AT**, α-1-antitrypsin; **CKD**, chronic kidney disease; **eGFR**, estimated glomerular filtration rate; **FFPE**, formalin-fixed paraffin-embedded; **FSGS**, focal segmental glomerulosclerosis; **GKD**, glomerular kidney disease; **GN**, Idiopathic glomerulonephritis; **IgAN**, IgA nephropathy; **MGN**, membranous glomerulonephritis; **SA**, sinapinic acid

*These authors contributed equally to the work.

Colour Online: See the article online to view Figs. 1–4 in colour.

Significance of the study

The body of work enclosed in this manuscript presents MALDI imaging as tool useful for detecting molecular signatures of glomerulonephritis and highlights the added value of correlating in situ proteomic information with urinary proteomics when attempting to identify translatable disease markers. Such molecular signatures could potentially assist in the routine diagnosis of idiopathic glomerulonephritis, where there is a need for less-invasive markers due to the potential complications and diagnostic difficulty associated with the renal biopsy. Specifically, the study employs MS imaging and immunohistochemistry to identify and validate one particular protein, α-1-antitrypsin (A1AT), which was found to be localized in the podocytes of sclerotic glomeruli. This finding suggests that A1AT may be implicated in the development of sclerotic lesions and could represent a marker of podocyte stress, an early sign of glomerulonephritis progression. Several fragments of this protein were also detected in urine and were shown to be overexpressed in the urine of patients who progressed to the latter stages of chronic kidney disease. Therefore, A1AT may represent a potential noninvasive proteomic indicator of the progression of glomerulonephritis.

1 Introduction

Glomerular kidney disease (GKD) represents the third leading cause of end-stage renal disease in Western countries [1]. Clinicians classify GKD in primary and secondary forms based on etiopathogenetic criteria. The former includes idiopathic glomerulonephritis (GN), such as membranous glomerulonephritis (MGN), focal segmental glomerulosclerosis (FSGS), and IgA nephropathy (IgAN), which represent the most frequent primary GKDs worldwide [2, 3]. Currently, the renal biopsy remains the gold standard for routine diagnosis [4]. However, the availability of pathological renal tissue also provides a great opportunity for the research of new diagnostic and prognostic markers that are potentially transferable into less invasive diagnostic tools if they are also detectable in biological fluids such as urine or serum. For this purpose, proteomic approaches are currently very promising and, among them, MALDI-MS imaging (MALDI-MSI) represents a potential technology that can be employed for biomarker discovery, providing the capability to match traditional morphological data related with pathology to proteomic information. Recent feasibility studies showed the possible application of MALDI-MSI in this field, showing the existence of alterations in the tissue protein profiles of MGN and minimal change disease patients [5]. Interestingly, these modifications were observed not only in tissue areas with evident pathological alterations, but also in regions with morphologically normal appearance, thus highlighting the potentiality of MALDI-MSI in nephropathology. In this complex background, we analyzed fresh-frozen tissue from heterogeneous forms of primary GN (FSGS, IgA, and MGN) by MALDI-MSI in order to investigate the potential application of this technique in GNs more deeply and explore the identification of possible new indicators of chronic kidney disease (CKD) progression.

2 Materials and methods

2.1 Materials

Conductive indium tin oxide glass slides, Protein Calibration Standard I and Peptide Calibration Standard I were purchased from Bruker Daltonik GmbH (Bremen, Germany). Sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), HPLC grade acetonitrile, ethanol, and TFA were purchased from Sigma-Aldrich Chemie (Buchs, Switzerland). Tissue-Tek® O.C.T. Compound was obtained from Sakura® Finetek (Alphen aan den Rijn, Netherlands). Alpha-1 antitrypsin (A1AT) polyclonal rabbit Ab was purchased from Dako (Glostrup, Denmark).

2.2 Tissue samples

The study included only primary GNs classified as idiopathic according to the strong clinical criteria presented in (Table 1). Fresh-frozen biopsies taken from patients, who underwent renal biopsy with a histological diagnosis of FSGS ($n = 6$), IgAN ($n = 6$), and MGN ($n = 7$), were collected. Normal cortical biopsies (controls, $n = 4$) corresponded to regions of kidney obtained from radical nephrectomy during tumour treatment. Control patients had no history of functional renal disease. The study was approved by the local Ethical Boards. Renal specimens were embedded in Tissue-Tek optimal cutting temperature within 30 min of biopsy execution or surgical procedure.

2.3 Sample preparation

For each specimen, two 4-μm-thick sections were cut and thaw mounted on the same conductive indium tin oxide glass slide (Bruker Daltonik GmbH) and stabilised for 30 min in a conventional dry oven at 85°C (Tecnovetro s.r.l., Monza,

Table 1. Clinical criteria used to determine the primitivity of MGN, FSGS and IgAN patients

	Criteria
MGN	- Antinuclear antibodies (ANA) below a titer of 1:80 - Negativity for anti-double stranded DNA antibodies - Absence of histological signs of lupus membranous nephritis (type V lupus nephritis) - Negative HBV and HCV serology - Negativity for malignancy
FSGS	- Serological negativity to HIV1, SV40, CMV, EBV, parvovirus B19 - No history of systemic hypertension - No renal dysgenesia/abnormalities - No history of drug abuse
IgAN	- Clinical presentation inconsistent for Henoch–Schoenlein purpura - Negative serology for HCV infection

Italy). The slides were then stored at −80°C until the day of analysis. Glass slides were thawed by desiccation for 30 min and then washed in a graded series of ethanol (70, 90, and 95%). Following the washes, they were again dried for 10 min via desiccation. SA was deposited on the slide through a sublimation apparatus (plate diameter 10 cm, S.B.L. Apparecchi Scientifici, Vimodrone, Italy) with 500 mg of SA at 145°C and 90 mTorr for 30 min [5]. Sublimation was followed by automated spraying of the SA (10 mg/mL in 60%/0.2% v/v acetonitrile/TFA) using the ImagePrep (Bruker Daltonik GmbH) to attain the desired matrix thickness. For the identification process, α-cyano-4-hydroxycinnamic acid (7 mg/mL in 50%/0.1% v/v acetonitrile/TFA) was deposited onto consecutive sections sectioned from the previously used renal specimens using the ImagePrep automated sprayer. The matrix was removed from the tissue by washing with a 50%/0.1% v/v acetonitrile/TFA solution. The resulting solution was concentrated using a HETO vacuum concentrator (Thermo Scientific, A. De Mou, Milano, Italy) for 30 min, to a final volume of 20 μL. A volume of 0.8 μL was spotted onto a Ground Steel MALDI Target Plate (Bruker Daltonik GmbH), allowed to dry, and then followed by an equal volume of 7 mg/mL α-cyano-4-hydroxycinnamic acid.

2.4 Mass spectrometric analysis

For MALDI-MSI analysis, all mass spectra were acquired in linear positive mode in the mass range of 3000–20 000 *m/z* using an UltrafleXtreme (Bruker Daltonik GmbH) MALDI-TOF/TOF MS equipped with a Smartbeam laser operating at 2 kHz frequency. External calibration was performed using a mixture of standard proteins within the mass range of 5500–17 000 *m/z* (ProtMix I, Bruker Daltonics). Images were acquired with a laser diameter of 50 μm and a rastering of 50 μm. For MALDI-MS/MS, representative mass spectra were acquired in reflectron positive mode in the mass range of 700–4500 *m/z*. Precursor ions were selected and fragmented using the laser-induced dissociation and LIFT™ technology and an MS/MS spectra obtained from the accumulation of ∼100 000 laser shots. External calibration was performed using a mixture of standard peptides within the mass range of 750–3500 *m/z* (PepMix I, Bruker Daltonics).

2.5 Histological evaluation

Following MALDI-MSI analysis, the matrix was removed with increasing concentrations of ethanol (70 and 100%) and the slides were stained using trichrome. The slides were then converted to digital format using a ScanScope CS digital scanner (Aperio, Park Center Dr., Vista, CA, USA) and pathological glomerular areas of interest (regions of interest) were highlighted by a pathologist, which included all of the glomeruli and regions manifesting in pathological alterations related to the disease. This allowed for the direct overlap of images and the integration of proteomic and pathological information. The study only compared the profiles glomerular tufts while tubulointerstitial features were not considered for the purpose of the current investigation; specific segmental glomerular areas of fibrosis were included. Globally sclerotic areas were excluded.

2.6 Data analysis

FlexImaging 3.0 (Bruker Daltonics) data, containing spectra of each entire measurement region, were imported into SCiLS Lab 2014 software (http://scils.de/; Bremen, Germany) after the acquisition. SCiLS was used to perform a series of preprocessing steps on the loaded spectra: baseline subtraction (TopHat algorithm) and normalization (total ion current algorithm). A series of further steps was performed in order to generate an average (avg.) spectrum representative of the whole measurement region and of the primary GN subclasses: peak picking (orthogonal matching pursuit algorithm), peak alignment (to align the detected ions with peak maxima), and spatial denoising (http://scils.de/; SCiLS Lab; 8.8 Spatial Denoising). PCA was also performed to reduce the high complexity of the data. Finally, ROC analysis was performed, with an AUC of >0.8 being required, as an

additional criteria to the $p < 0.05$, for a peak to be considered as statistically significant. For MALDI-MS/MS spectra, baseline subtraction and smoothing were performed using FlexAnalysis 3.4 (Bruker Daltonics). All MS/MS spectra were searched against the Swiss-Prot database (Release 2015_05 of Apr 29, 2015) with the Mascot 2.4 search engine (Matrix Science, London, UK). Mass tolerances were set at 2.5–3 Da for MS and 1.4 Da for MS/MS. No enzymes or any fixed post-translational modifications were set in the search parameters.

2.7 Immunohistochemistry with A1AT antibody

In order to confirm the proteomic identification data, a further series of formalin-fixed paraffin-embedded (FFPE) renal biopsies taken from patients with IgAN and FSGS were tested for IHC analysis. This validation group included IgAN with wide sclerosis ($n = 4$), IgAN with a prevalent mesangioproliferative pattern without sclerosis ($n = 4$), and FSGS ($n = 6$). For each specimen, 3 μm thick sections were cut from FFPE blocks. After deparaffinization and rehydration, slides underwent endogenous peroxidase blockage and an antigen retrieval process via EnVision Flex (DAKO). Finally, Autostainer Link 48 (Dako) was used to apply the primary antibody directed against A1AT (polyclonal rabbit, Dako).

3 Results

3.1 Proteomic signatures of primary GN

Initially, the average mass spectra of the entire cohort of patients affected by GN was built within the 3000 to 15 000 m/z mass range. Several of the signals (m/z) present in the spectra had a statistically significant higher intensity ($p < 0.05$ and AUC > 0.8) in primary GN, most specifically signals at m/z 4025, 4048, and 4963, compared with controls (Fig. 1). Moreover, when the specific protein profiles of FSGS, IgAN, and MGN were compared, several signals showed different intensity (Fig. 2A–C). Among them, the intensity of the two signals at m/z 4025 and 4048 was significantly higher in FSGS in comparison to IgAN and MGN (AUC values of 0.84 and 0.82, respectively, Fig. 2A–C). Additionally, the specific protein profile of IgAN showed two signals at m/z 4963 and 5072 with a higher intensity when compared to FSGS, and a higher intensity of signals at m/z 5072 and 6180 when compared to MGN (Fig. 2A–C). Unsupervised PCA was performed on the entire dataset in order to further highlight any proteomic differences between FSGS, MGN, and IgAN, (Fig. 3A). In general, the spectra related to FSGS and IgAN presented distinct distributions, except for one IgAN case (Fig. 3A). The coregistration of the proteomic findings with the histological image highlighted that the group of spectra present in this IgAN patient, which were in common with the spectra from the FSGS cohort, were localized to a distinct group of three sclerotic glomeruli. Therefore, we proceeded with virtual microdissection in order to export spectra from these particular glomeruli and PCA was again performed (Fig. 3B). The spectra exported from these sclerotic glomeruli found within this IgAN patient showed a similar distribution to the entire FSGS cohort in the PCA score chart. In fact, they also showed similar protein profiles, thus strengthening the initial observation that MALDI-MSI has the capability to distinguish individual lesions or subregions of tissue even within an individual form of the studied primary GN. Interestingly, the same IgAN patient identified as more comparable with the FSGS cohort following the PCA presented a similar level of intensity of the two target signals (m/z 4025 and 4048) previously detected as upregulated in FSGS patients (Fig. 2E). Upon viewing the spatial localization of these two signals, they were again correlated with the previously histologically identified sclerotic regions (Fig. 4). Furthermore, these ions also shared

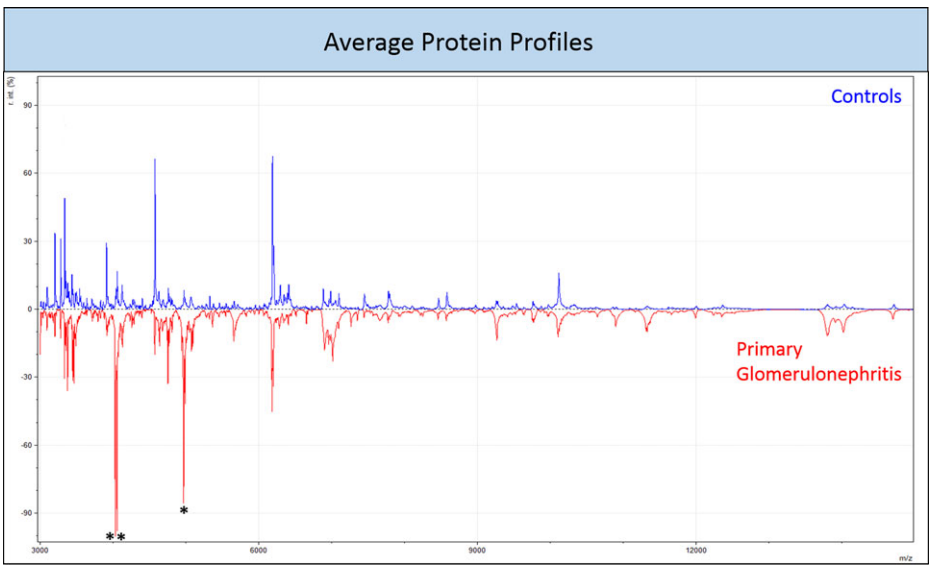

Figure 1. Average protein profiles of healthy renal tissue (top) and from patients with primary glomerulonephritis (bottom) in the 3–15 000 m/z mass range.

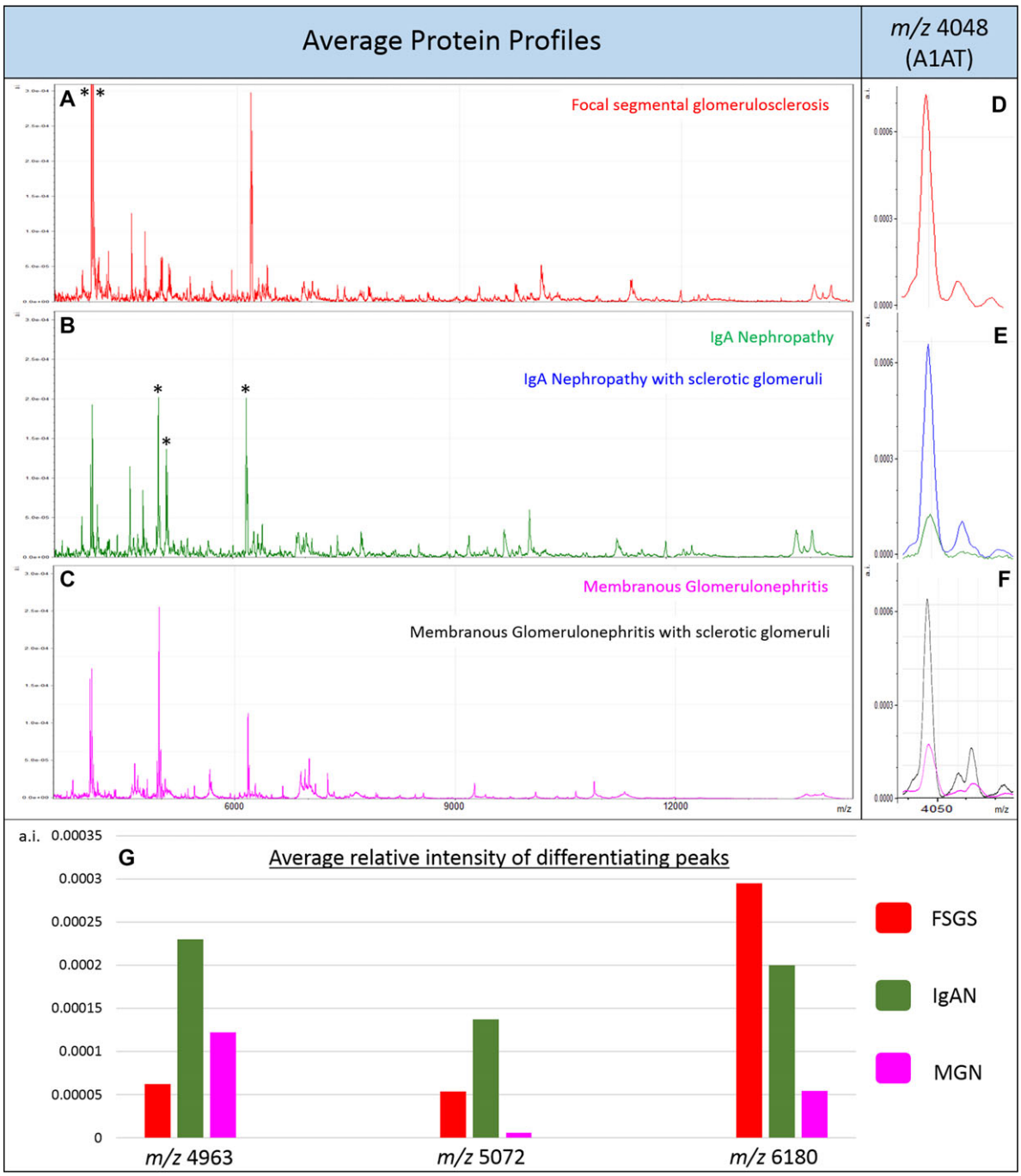

Figure 2. (A–C) Average protein profiles of patients with FSGS (A), IgAN (B), and MGN (C) in the 3–15 000 *m/z* mass range with differentiating peaks (AUC > 0.8) denoted by an asterik. (D–F) Relative intensity of *m/z* 4048 (A1AT) in FSGS (D), IgAN and IgAN with sclerotic glomeruli (E; green and blue, respectively) and MGN and MGN with sclerotic glomeruli (F; fuchsia and black, respectively). (G) The average relative intensity for the three further differentiating peaks (*m/z* 4963, 5072, and 6180) in FSGS, IgAN, and MGN.

a specific sclerosis-related localization within FSGS biopsies (Fig. 4). More interestingly, there was also an individual patient within the MGN group that showed a higher intensity of these signals when compared to the other MGN patients (Fig. 2F). These signals were again well correlated with sclerotic areas within the tissue of this patient.

3.2 Identification of A1AT

The two signals at *m/z* 4025 and 4048 had the capability to discriminate sclerotic glomeruli within different forms of primary GN. Therefore, their identity was investigated by acquiring MS/MS spectra by MALDI-TOF/TOF.

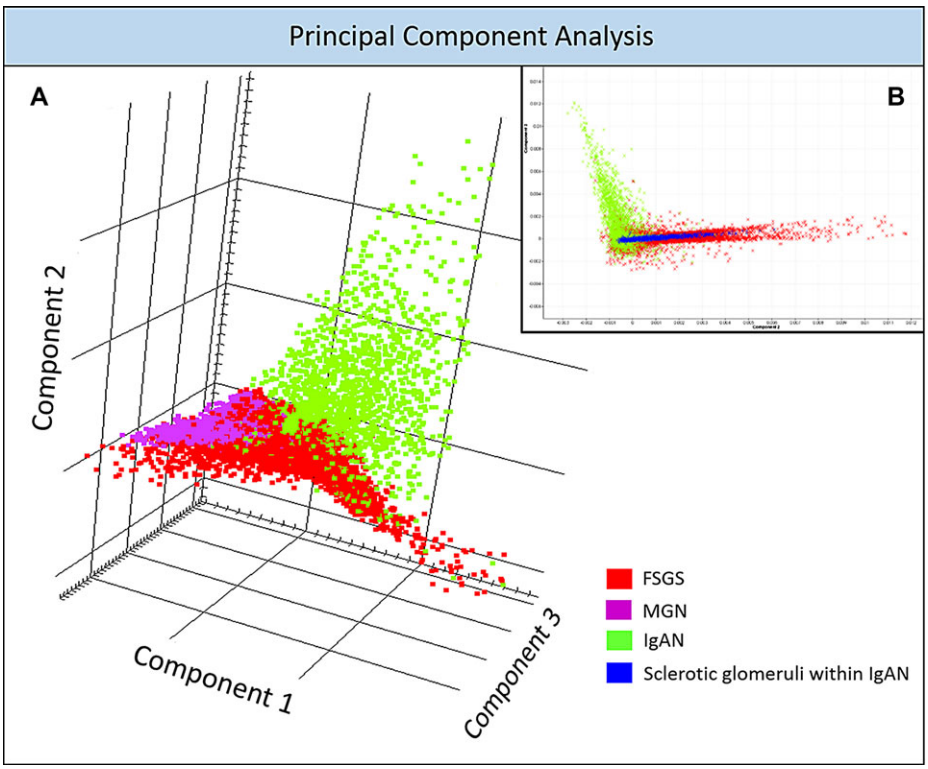

Figure 3. (A) Three-dimensional principal component analysis score chart presenting the distribution of spectra from FSGS (red), MGN (fuchsia), and IgAN (green) patients. (B) Two-dimensional principal component analysis score chart presenting the distribution of spectra from FSGS (red) and IgAN (green) patients along with the distribution of spectra virtually microdissected from sclerotic glomeruli within an IgAN patient (blue).

The peptide fragments in the resulting spectrum were searched against the Swiss-Prot database with the Mascot 2.4 search engine. The signal at *m/z* 4048 was identified as a peptide fragment of A1AT (IPPEVKFNKPFVFLMIEQNTK-SPLFMGKVVNPTQK) with a Mascot score of 77. The IHC staining for A1AT on renal biopsies showed a diffuse positivity among sclerotic areas of FSGS and IgAN and scant positivity/negativity among IgAN with a prevalent mesangioproliferative pattern and controls. Interestingly, in renal biopsies classified as "sclerotic," there was also a strong positivity to A1AT in the cytoplasm of podocytes (Fig. 4). On the contrary, the podocytes present in the renal biopsies with a mesangioproliferative pattern of IgAN and normal glomeruli were completely negative, suggesting that

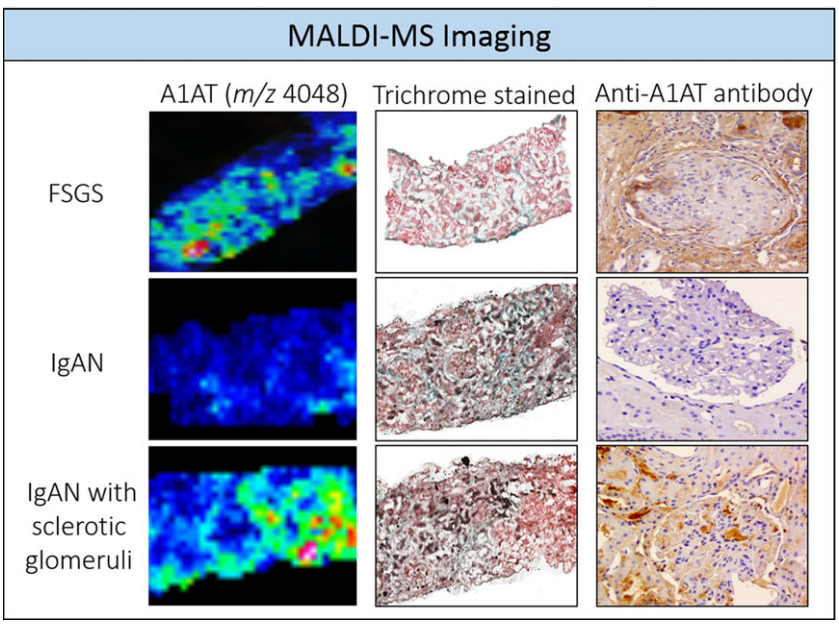

Figure 4. (Left) The molecular distribution of *m/z* 4048 (A1AT) in sections of bioptic renal tissue, obtained using MALDI-MSI, (center) trichrome-stained image of the identical sections used for MALDI-MS imaging and (right) immunohistochemical staining with the anti-A1AT antibody from a validation set of patients. The collection of images is derived from patients with FSGS (top), IgAN (middle), and IgAN with sclerotic glomeruli (bottom).

intraepithelial deposits of A1AT are consistent with a dysfunction of podocytes.

4 Discussion

MALDI-MSI could be the ideal tool for a new approach in investigating GN, both for diagnostic and prognostic purposes. This technique has been reported to be capable of providing specific proteomic profiles for the physiological counterpart and for pathological glomeruli [5]. It can define nosological entities, such as IgAN and FSGS, through their distinctive signatures and could be potentially useful in the biological comprehension of these lesions, supporting the existence of specific molecular alterations. The characterization of the proteomic phenotype of glomeruli is mainly related to the possible clinical development of biomarkers for the prognostic stratification of patients with CKD progression. MALDI-MSI is the newest and most promising imaging method for combining protein/peptide expression with distinct localization inside tissue. In this study, we provide additional evidence of the capability of this technology to discriminate normal kidney from pathological GN, based on specific signals (m/z 4025, 4048, and 4963; Fig. 1) that may represent indicators of CKD development. Moreover, specific disease-related signatures (m/z 4025 and 4048 for FSGS, m/z 4963 and 5072 for IgAN, Fig. 2) were also found. MALDI-MSI showed a common proteomic profile overlap between IgAN and FSGS in sclerotic glomerular regions (Fig. 3). In particular, two specific ions at m/z 4025 and at m/z 4048 were of higher intensity in the sclerotic areas of all FSGS patients as well as in the single IgA patient that presented sclerotic lesions (Fig. 4).

The signal at m/z 4048 was identified by MALDI-TOF/TOF as the resulting peptide fragments belonged to the A1AT protein. A1AT is a major serine proteinase inhibitor (serpin) found in human plasma. It is a glycoprotein with a broad range of activities, including the downregulation of neutrophil elastase during the inflammatory processes. Kwak et al. recently evaluated this protein expression in renal biopsies [6], while other authors independently described its overexcretion in the urine of some patients with primary GNs, most specifically FSGS, IgAN, and minimal change disease [7–9]. The IHC staining against A1AT demonstrated its localization in the cytoplasm of podocytes present in sclerotic glomeruli. This represented an intriguing finding related with possible epithelial dysfunction and consequent insufficient degradation of the extracellular matrix. In this setting, many studies correlated the development of FSGS both with an excessive loss [10] and a hypertrophy of podocytes [11] and A1AT could be one of the markers of podocyte stress, in addition to nephrin, Wilms tumor antigen 1 (WT1), the glomerular epithelial protein 1 (GLEPP1), and thymosin beta-4 [12].

Additionally, the identification of A1AT fragment(s) in urine samples taken from GN patients has been reported [13, 14]. Smaller fragments of the peptide derived from the A1AT protein that we have identified in tissue were also previously detected in urine samples taken from CKD patients. In 2010, Good et al. investigated the urinary peptidome of 230 patients with renal disease and compared them to 379 healthy controls. The authors detected 273 urinary peptides found to be significantly different between cases and controls. Using support vector machines, those potential biomarkers were integrated into a single classifier, called "the CKD 273 classifier," which was validated in several studies for the diagnosis and prognosis of CKD. For three of the detected differentiating ions (m/z 4963, 5072, and 6180), peaks of similar molecular weight were detected in the CKD 273 classifier (personal communication). However, focusing on A1AT, 18 different fragments of this protein were present in the CKD 273 classifier. All of them were upregulated in CKD patients in comparison to the control groups. Five of these fragments partially overlapped with the A1AT fragment we identified in tissue samples. Moreover, urinary A1AT fragments were also found to be correlated with CKD progression in Schanstra et al. The authors of this study performed de novo correlation analysis to investigate which urinary peptides were highly associated with CKD progression (high progression patients were defined on the basis of a decline in percentage of estimated glomerular filtration rate (% eGFR) slope/year $>-5\%$). Thirty-five urinary fragments of A1AT were found to be correlated with baseline eGFR and % eGFR slope/year. Among them, four fragments partially overlapped with the A1AT fragment that we identified in tissue. Moreover, the same A1AT fragment was found to be uprepresented in the urine of another cohort of patients classified as CKD progressors with respect to nonprogressors (data not shown). In this setting, the combined findings from tissue MALDI-MSI and urinary peptidomics provide further agreement with studies that describe the presence of glomerulosclerosis and the aforementioned podocyte changes [10, 11] as early signs of disease progression [15, 16]. Thus, the consistency between tissue and urinary results suggests that A1AT should be further investigated as a putative noninvasive biomarker of CKD progression by studying both bioptic renal tissue and urine from the same patients within a very well-defined cohort that is based upon carefully selected etiologies (FSGN, IgAN, etc.).

In conclusion, this study shows a promising application of MALDI-MSI in the discovery of biomarkers for the assessment of CKD progression. MALDI-MSI not only facilitates the analysis of fresh-frozen specimens but also FFPE tissue, making retrospective studies possible [17]. More specifically, this technology could translate molecular knowledge obtained directly in tissue into routine clinical practice, such as the successful application of the CKD 273 classifier that is based on CE–MS tools.

Furthermore, the localization of A1AT within the sclerotic glomeruli, as highlighted by IHC, reveals that this protein could be related with the so-called "podocyte stress theory" [18–20] and the emerging fibrogenic role of different biomarkers in glomerulosclerosis [11, 12]. Due to some limitations of our study related with the small number of cases analyzed,

further similar studies are needed for a definitive confirmation of this hypothesis and to validate the role of A1AT in GKDs.

This work was supported by grants from the MIUR: FIRB 2007 (RBRN07BMCT_11), FAR 2010–2014; from iMODE-CKD (FP7-PEOPLE-2013-ITN); and in part by the COST Action (BM1104) Mass Spectrometry Imaging: New Tools for Healthcare Research.

The authors have no other relevant affiliation or financial involvements with any organisation or entity with a financial interest in or financial conflict with the subject matter or material discussed in the manuscript apart from those disclosed.

Claudia Pontillo is employed by Mosaiques in the course of a Marie Curie programme and she has no other relevant affiliation or financial involvements with any organisation or entity with a financial interest in or financial conflict with the subject matter or material discussed in the manuscript apart from those disclosed.

5 References

[1] U.S. Renal Data System, USRDS 2013 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. Bethesda, MD 2013.

[2] Wyatt, R. J., Julian, B. A., IgA nephropathy. *N. Eng. J. Med.* 2013, *368*, 2402–2414.

[3] Orth, S. R., Eberhard, R., The nephrotic syndrome. *N. Eng. J. Med.* 1998, *338*, 1202–1211.

[4] Pagni, F., Galimberti, S., Goffredo, P., Basciu, M. et al., The value of repeat biopsy in the management of lupus nephritis: an international multicentre study in a large cohort of patients. *Nephrol. Dial. Transplant.* 2013, *28*, 3014–3023.

[5] Mainini, V., Pagni, F., Ferrario, F., Pieruzzi, F. et al., MALDI imaging mass spectrometry in glomerulonephritis: feasibility study. *Histopathology* 2014, *64*, 901–906.

[6] Kwak, N. J., Wang, E. H., Heo, I. Y., Jin, D. C. et al., Proteomic analysis of alpha-1-antitrypsin in immunoglobulin A nephropathy. *Proteomics Clin. Appl.* 2007, *1*, 420–428.

[7] Navarro-Muñoz, M., Ibernón, M., Bonet, J., Pérez, V. et al., Uromodulin and α(1)-antitrypsin urinary peptide analysis to differentiate glomerular kidney diseases. *Kidney Blood Press. Res.* 2012, *35*, 314–325.

[8] Graterol, F., Navarro-Muñoz, M., Ibernón, M., López, D. et al., Poor histological lesions in IgA nephropathy may be reflected in blood and urine peptide profiling. *BMC Nephrol.* 2013, 14:82. doi: 10.1186/1471-2369-14-82.

[9] Pérez, V., Ibernón, M., López, D., Pastor, M. C. et al., Urinary peptide profiling to differentiate between minimal change disease and focal segmental glomeurlosclerosis. *PLoS One* 2014, *9*, e87731. doi: 10.1371/journal.pone.0087731.

[10] Lemley, K. V., Lafayette, R. A., Safai, M., Derby, G. et al., Podocytopenia and disease severity in IgA nephropathy. *Kidney Int.* 2002, *61*, 1475–1485.

[11] Wiggins, J. E., Goyal, M., Sanden, S. K., Wharram, B. L. et al., Podocyte hypertrophy, "adaptation," and "decompensation" associated with glomerular enlargement and glomerulosclerosis in the aging rat: prevention by calorie restriction. *J. Am. Soc. Nephrol.* 2005, *16*, 2953–2966.

[12] Xu, B. J., Shyr, Y., Liang, X., Ma, L. J. et al., Proteomic patterns and prediction of glomerulosclerosis and its mechanisms. *J. Am. Soc. Nephrol.* 2005, *16*, 2967–2975.

[13] Good, D. M., Zurbig, P., Argiles, A., Bauer, H. W. et al., Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. *Mol. Cell. Proteomics* 2010, *9*, 2424–2437.

[14] Schanstra, J. P., Zurbig, P., Alkhalaf, A., Argiles, A. et al., Diagnosis and prediction of CKD progression by assessment of urinary peptides. *J. Am. Soc. Nephrol.* 2015, *26*, 1999–2010.

[15] Barton, M., Tharaux, P. L., Endothelin and the podocyte. *Clin. Kidney J.* 2012, *5*, 17–27.

[16] Kriz, W., Lemley, K. V., A potential role for mechanism forces in the detachment of podocytes and the progression of CKD. *J. Am. Soc. Nephrol.* 2015, *26*, 258–269.

[17] De Sio, G., Smith, A. J., Galli, M., Garancini, M. et al., A MALDI-mass spectrometry imaging method applicable to different formalin-fixed paraffin-embedded human tissues. *Mol. Biosyst.* 2015, *11*, 1507–1514.

[18] Smeets, B., Moeller, M. J., Parietal epithelial cells and podocytes in glomerular diseases. *Semin. Nephrol.* 2012, *32*, 357–367.

[19] Moeller, M. J., Smeets, B., Role of parietal epithelial cells in kidney injury: the case of rapidly progressing glomerulonephritis and focal and segmental glomerulosclerosis. *Nephron Exp. Nephrol.* 2014, *126*, 97–100.

[20] Kretzler, M. Role of podocytes in focal sclerosis: defining the point of no return. *J. Am. Soc. Nephrol.* 2005, *16*, 2830–2832.

6.4 Curriculum vitae: my curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

6.5 List of publications:

Original research articles

1.) Pontillo C, Lotte Jacobs, Staessen JA, Schanstra JP, Rossing P, Heerspink HJL, Siwy J, Mullen W, Vlahou A, Mischak H, Vanholder R, Zürbig P, Jankowski J. A urinary proteome-based classifier for the early detection of decline in glomerular filtration. *NDT,2016* (IF: 3.57)

2.) Siwy J, Zürbig P, Argiles A, Beige J, Haubitz M, Jankowski J, Julian B A, Linde P G, Marx D, Mischak H, Mullen W, Novak J, Ortiz A, Persson F, **Pontillo C**, Rossing P, Rupprecht H, Schanstra J P, Vlahou A, Vanholder R, Non-invasive diagnosis of chronic kidney diseases using urinary proteome analysis Nephrology Dialysis Transplantation, 2016 (IF: 3.57)

3.) Lindhardt M, Persson F, Currie G, **Pontillo C**, Beige J, Delles C, von der Leyen H, Mischak H, Navis G, Noutsou M, Ortiz A, Ruggenenti PL, Rychlik I, Spasovski G, Rossing P. Proteomic prediction and Renin angiotensin aldosterone system Inhibition prevention Of early diabetic nephRopathy in TYpe 2 diabetic patients with normoalbuminuria (PRIORITY): essential study design and rationale of a randomised clinical multicentre trial. BMJ Open. 2016 Mar (IF: 2.97)

4.) Smith A, L'Imperio V, De Sio G, Ferrario F, Scalia C, Dell'Antonio G, Pieruzzi F, **Pontillo C**, Filip S, Markoska K, Granata A, Spasovski G, Jankowski J, Capasso G, Pagni F, Magni F. α-1-Antitrypsin detected by MALDI imaging in the study of glomerulonephritis: Its relevance in chronic kidney disease progression. Proteomics. 2016 Jun (IF: 1.56)

Reviews and Editorial

1.) Pontillo C, Filip S, Borràs DM, Mullen W, Vlahou A, Mischak H. CE-MS-based proteomics in biomarker discovery and clinical application. Proteomics Clin Appl. 2015 (IF: 2.96)

2.) Filip S, **Pontillo C**, Peter Schanstra J, Vlahou A, Mischak H, Klein J. Urinary proteomics and molecular determinants of chronic kidney disease: possible link to proteases. Expert Rev Proteomics. 2014 (IF: 2.89)

3.) Pontillo C, Mischak H. Urinary biomarkers to predict CKD: the future is in multimarker panels? NDT, January 2016 (IF: 3.57)

6.6 Acknowledgements

I would like to thank all the people that contributed to this work. In particular, I would like to express my special thanks and appreciation to:

Prof. Dr. Joachim Jankowski for all the support and assistance as well as for the correction of publications and thesis

Prof. Dr. Harald Mischak to give me the opportunity to develop my project as well as for all the guidance and advices in any aspects of my PhD

Dr. Joost Schanstra to guide me to critically evaluate my research and for many interesting discussions on biomarkers

Dr. Petra Zürbig for helping me in the evaluation of CE-MS data

Dr. Thomas Koeck for all the support, guidance and advices

Dr. Lotte Jacobs and Prof. Staessen for the great help with statistical analysis

Dr. Adela-Ramirez Torres, Dr. Maria Frantzi and Ms. Agnieszka Latosinska for the encouraging discussions on proteomics, for the support in any scientific aspects as well as to be my friends and examples of great scientists

All the people working at Mosaiques Diagnostics to be present in any aspects of the project

All the partners and PhD students of the iMODE-CKD consortium for many interesting discussions and exciting meetings and collaborations