Peptides in Plasma, Urine, and Dialysate: Toward Unravelling Renal Peptide Handling

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1. Introduction

Proteins are the fundamental building blocks of life and the functional unit for biochemical reactions. Pathological changes in protein structure, expression level, or activity can lead to disease(s). Based on this theorem, diseases can be studied via the investigation of proteomics/peptidomics changes,[1] with the help of recent advances in proteomics and peptidomics.[2–3] Biofluids such as urine, plasma, and spent hemodialysate (a mixture of technical HD fluid and patient’s ultrafiltrate) are rich sources for investigation,[4,5] and highly relevant in kidney disease.[6–8] In particular, peptides in spent hemodialysate may be valuable biomarkers to guide the treatment of dialysis patients: for example, deficiency of vitamin D-binding proteins in dialysis was reported to be associated with poor survival.[9,10] Therefore, a better understanding of the flux of peptides may also help improving care for ESRD patients, to strike a balance between the clearance of toxic substances and retention of beneficial molecules. Although “peptidomics” and “proteomics” are often used interchangeably, we refer our analysis as peptidomics because we study the undigested, naturally occurring endogenous peptides in the biofluids. Unlike proteomics, enzymatic/chemical digestion is typically not required for peptidomics analysis.[11,12]

From a physiological point of view, peptides in these three biofluids should be, to a large extent, connected. Over 1700 L of blood, equivalent to one-fifth of the cardiac output, is filtered daily by the renal glomeruli via hydrostatic pressure. At a normal glomerular filtration rate (GFR) of 120 mL min−1, this process forms around 170 L of ultrafiltrate. The ultrafiltrate then undergoes selective reabsorption in the renal tubule, so that the subsequent volume of urine drops to ≈1–2 L.[13] The urine is collected in the calyx and via renal pelvis as the ureter enters the bladder, where it is stored for a few hours before voiding. In comparison, the spent hemodialysate is a simplified version of the ultrafiltration fluid, produced using a dialysis membrane (an artificial kidney), with tubular reabsorption missing.[14] It also lacks the secretory proteins from the kidney and urinary tract,[15] and some middle-to-high molecular weight proteins may be depleted due to adsorption on dialysis membranes.[16] The differences in peptide handling between hemodialysis and kidney are graphically illustrated in Figure 1.
The proteomic content of spent hemodialysate and its relationship with urine and blood are not well described. A study from Kaiser et al. suggests a minor overlap between the proteome of urine and spent hemodialysate,[17] in agreement with the findings presented by the European Uremic Toxin Working Group (EUTox).[18] A recent study by Magalhães et al. compared the peptidomics content of urine and plasma, revealing no correlation between the two peptidomes, except for collagen-based peptide fragments where a more pronounced overlap was detectable. The authors suggest that selective tubular reabsorption could account for the difference in the resulting peptidomes.[19] Pedrini et al. identified 277 proteins from spent hemodialysate, among them the most abundant are those with known uremic effect, such as complement factor D, β2-microglobulin, retinol-binding protein 4, and myoglobin. However, the authors only assessed the tryptic peptides, but not the endogenous.[20] Our exploratory study was based on the hypothesis that hemodialysis replaces glomerular filtration and aims at gaining first insight into the comparative distribution of the peptidome in these body fluids. This information may help to better understand the processes of filtration and reabsorption in the kidney, so that a renal replacement regime that better mimics the functionality of a kidney could be designed.

### 2. Results

#### 2.1. Peptidome Profile of Spent Hemodialysate

In 15 samples, we detected a total of 1727 unique peptides (on average 755 peptides per sample) and obtained sequence information of 352 (20.4%) from them. The sequenced peptides covered 55.4% of the total detected peptide signal. The 20 most abundant peptides from spent hemodialysate are listed in Table S2A, Supporting Information. These were β2-microglobulin, thymosin β4, and fragments from fibrinogen α, collagen type I, and III, and from serum amyloid A-1.

When grouping the peptides according to their parental proteins/peptides, β2-microglobulin accounted for the strongest combined signal (3 peptides, 51.8% of the total peptide signal), followed by thymosin β4 (5 peptides, 24.4%), collagen alpha-1(I) (COL1A1) (123 peptides, 8.2%), and fibrinogen alpha (12 peptides, 5.8%). We identified five albumin fragments, which accounted for 0.06% of the total peptide signal. No fragments from uromodulin were detected (Table 1A).

#### 2.2. Urine Peptidome

On average 1131 peptide signals were detected per sample. When combined, a total of 6278 urinary peptides were detected, 1580 (25.2%) of these could be sequenced, accounting for 74.3% of the total detected signal. As listed in Table 2B, Supporting Information, fragments of albumin were the most abundant peptides in the urine of CKD patients. Other abundant peptides were derived from α1-antitrypsin, COL1A1, fibrinogen α, and β2-microglobulin. As presented in Table 1B, albumin (49 fragments) alone was responsible for 45.7% of the total peptide signal, followed by α1-antitrypsin (79 fragments, 14.4%), COL1A1 (319 fragments, 11.7%), and fibrinogen α (30 fragments, 4.9%). Other prominent peptides included β2-microglobulin (3.9%) and uromodulin (1.8%).

#### 2.3. Plasma Peptidome

A total of 1743 unique endogenous peptides were detected in 15 plasma samples. Of these, we could sequence 419 (24.0%) peptides, which accounted for 29.7% of the total detected peptide signal. The 20 most abundant peptides are listed in Table 2C, Supporting Information. The proteins of origin of these peptides are more heterogeneous than the results obtained from urine or spent dialysate. We detected 18.8% COL1A1 (113 fragments), 16.5% fibrinogen alpha chain (20 fragments), and nine fragments from α1-antitrypsin (0.7% of the total signal), and six from thymosin β4 (2.2%; Table 1C).
Table 1. Distribution of sequenced peptides from A) Spent hemodialysate, B) urine, and C) plasma samples from CKD patients and D) urine samples of normal albuminuria individuals. Peptides are sorted accounting to frequency. The relative abundance of peptide(s) (%) was calculated by Mean abundance × 100/Abundance of all peptides in a given cohort. Only peptides that were among the four most abundant peptides in at least one body fluid are presented. COL1A1: collagen alpha-1(I) chain.

|                | Number of identified peptides | Relative abundance [%] |
|----------------|------------------------------|------------------------|
| **A) In Spent hemodialysate (CKD)** |                              |                        |
| COL1A1         | 123                          | 8.2                    |
| Fibrinogen alpha chain  | 12                           | 5.8                    |
| Albumin        | 5                            | 0.06                   |
| Thymosin beta-4 | 5                            | 24.4                   |
| Alpha-1-antitrypsin | 3                           | 0.03                   |
| Beta-2-Microglobulin  | 3                            | 5.18                   |
| Uromodulin     | 0                            | 0                      |
| Others         | 201                          | 10.0                   |
| **B) In urine (CKD)** |                              |                        |
| COL1A1         | 319                          | 11.7                   |
| Alpha-1-antitrypsin | 79                          | 14.4                   |
| Beta-2-Microglobulin  | 53                           | 3.9                    |
| Albumin        | 49                           | 45.7                   |
| Fibrinogen alpha chain  | 30                           | 4.9                    |
| Uromodulin     | 27                           | 1.8                    |
| Thymosin beta-4 | 4                            | 0.3                    |
| Others         | 1019                         | 17.1                   |
| **C) In plasma (CKD)** |                              |                        |
| COL1A1         | 113                          | 18.8                   |
| Fibrinogen alpha chain  | 20                           | 16.5                   |
| Beta-2-Microglobulin  | 10                           | 0.2                    |
| Albumin        | 9                            | 0.7                    |
| Thymosin beta-4 | 6                            | 2.2                    |
| Uromodulin     | 2                            | 0.2                    |
| Others         | 252                          | 25.0                   |
| **D) In urine (normal albuminuria)** |                              |                        |
| COL1A1         | 614                          | 46.9                   |
| Alpha-1-antitrypsin | 79                          | 0.1                    |
| Beta-2-Microglobulin  | 69                           | 0.004                  |
| Albumin        | 52                           | 0.03                   |
| Fibrinogen alpha chain  | 51                           | 4.7                    |
| Uromodulin     | 46                           | 13.9                   |
| Thymosin beta-4 | 8                            | 0.05                   |
| Others         | 2109                         | 34.3                   |

2.4. Comparisons of the Peptidome Profiles

We identified 161 common peptides in three body fluids, at the same time 37, 1161, and 110 peptides were unique in spent hemodialysate, urine, and plasma, respectively, in our analysis (Figure 2). We listed all sequenced peptides in all three body fluids with their rank by mean abundance in Table S3, Supporting Information. COL1A1-derived peptides were the most frequently detected peptides in all three body fluids. In spent hemodialysate and plasma, fibrinogen alpha-derived peptides were the second most frequent; while in urine, the second most frequent was from α1-antitrypsin. β2-microglobulin and thymosin β4 were the two most abundant peptides in Spent hemodialysate. These two peptides could also be detected in urine and plasma. The most abundant peptide in urine was from albumin, which was present but at a significantly lower level in plasma and spent hemodialysate. When examining the correlation of mean peptide abundance between the three peptidomes (Figure 3), a significant correlation between the peptidomes of Spent hemodialysate and urine ($R = 0.56$, $p$-value $= 8.4 \times 10^{-27}$), and between spent hemodialysate and plasma with ($R = 0.28$, $p$-value $= 8.4 \times 10^{-5}$) was detectable. However, no significant correlation ($R = 0.82$, $p$-value $= 0.15$) was found between plasma and urine peptidomes, in agreement with a previous study.[19]

2.5. Reference to the Peptidomics from a Normal albuminuria Population

Since the urine samples investigated in this study were from patients with advanced-stage CKD (for comparability with patients on dialysis), the impact of proteinuria in the urine analysis could not be avoided and appeared obvious. To assess the potential influence of proteinuria, we compared the urinary peptidomics data with those from the general population, obtained from the FLEMENGHO study, which consists of 777 non-proteinuric urine samples.[25] In this cohort, we identified 3154 urinary peptides. The peptides are listed in Table S3, Supporting Information, ranked according to mean abundance.
Figure 3. Correlation of the mean peptide abundance of overlapping peptides in spent hemodialysate, plasma, and urine. The peptide abundances were ln-transformed.

As listed in Table 2D, the peptides were predominantly from COL1A1 (614 fragments, 46.9% of total peptide signal) and uromodulin (46 fragments, 13.9%), while the signal from albumin plunged to 0.3% in comparison to the peptidome of CKD patients (45.7%). Among the 20 most abundant peptides (Table 2D, Supporting Information), fragments from albumin were no longer present. The most abundant peptide fragments came from uromodulin (3/20), fibrinogen $\alpha$ chain (1/20), collagen type III (4/20), and predominantly collagen type I (12/20), in very good agreement with previous studies.\[22\]

The comparison of the 20 most abundant peptides between the normal albuminuria and CKD subjects is presented in Figure S1, Supporting Information. While the distribution of the 20 most abundant peptides from subjects with preserved kidney function remained relatively unchanged in the urine of the CKD patients (Figure S1, Supporting Information, right), the most abundant urinary peptides in CKD subjects were found only at highly reduced levels in the controls. These included eight albumin, four alpha1-antitrypsin, two B2-microglobulin, and two fibrinogen alpha chain peptide fragments (Figure S1, Supporting Information, left).

Most of the results obtained were as expected, however, the very high abundance of thymosinβ4 in spent hemodialysate was surprising and not reported previously. We therefore further examined the correlation between the abundance of thymosinβ4 and eGFR in 2289 patients from the urine proteome database.\[26\] As shown in Figure 4, a highly significant negative association of thymosinβ4 abundance with eGFR ($R = -0.39$, p-value $= 3.9 \times 10^{-81}$) was detected, which is exacerbated upon kidney failure.

3. Discussion

The urine and plasma peptidome of 15 CKD patients and spent hemodialysate from 13 patients were evaluated to shed light on the kidney peptide handling. Overall, we identified 352, 1580, and 419 peptide fragments in spent hemodialysate, urine, and plasma, respectively. The higher number of peptides in urine is likely the result of a higher concentration due to tubular activity, reabsorption of water, and consequently increase in the concentration of compounds not being reabsorbed with similar efficiency.

To our knowledge, this is the first study to demonstrate a correlation between spent hemodialysate, urine, and plasma. Surprisingly, no similarity between urine and plasma could be found, although we found the expected similarity between urine and spent hemodialysate as well as between plasma and spent hemodialysate (Figure 3). Because both spent hemodialysate and urine are derived from plasma, the difference in correlation with plasma is likely due to differences in the mechanics of the kidney and dialysis membrane. As illustrated in Figure 1, reabsorption and secretion that are unique to the kidney but absent in hemodialysis could be the plausible sources of such difference. In addition, urinary peptides are subjected to the activity of kidney-specific proteases, not present in circulation, relevant for peptides in both plasma and spent hemodialysate.

We confirmed the presence of peptides in spent hemodialysate documented in the literature, including α1-antitrypsin, albumin, apolipoprotein A-IV, B2-microglobulin, fibrinogen α chain, gelatin, insulin-like growth factor II, Ig kappa chain C region, osteopontin, and thymosinβ4.\[14\] Among them, B2-microglobulin and thymosin β4 were the two most abundant peptides in spent hemodialysate. B2-microglobulin, via interactions with other proteins, fosters the deposition of stable amyloid-like complexes in bones, tissues, vessels, and heart.\[27\] Therefore, an elevated concentration of B2-microglobulin in circulation may provoke deterioration of renal function in combination with adverse cardiovascular outcomes in CKD patients.

Thymosinβ4 was reported as potentially beneficial in CKD by regulating fibrosis and inflammation.\[28\] We found a highly significant negative correlation between urinary thymosinβ4 and eGFR (Figure 4), in agreement with the high level of thymosinβ4 detected in spent hemodialysate. We could only detect a moderate level of thymosin β4 in two of the 15 plasma samples that we analyzed. This is consistent with the literature description that concentration of thymosin β4 in plasma was <1% of its...
concentration in the whole blood.\textsuperscript{[29]} Studies in transgenic mice models suggested that endogenous thymosin β4 is essential for kidney health, while a lack of endogenous thymosin β4 worsens glomerular disease and angiotensin-II-induced renal injury in mice.\textsuperscript{[10]} Combining our finding that urinary thymosin β4 is negatively associated with kidney function, it appears possible that increased urinary excretion of thymosin β4, consequently a loss of thymosin β4, is associated with CKD severity. Alternatively, the observed increase in urinary thymosin β4 in advanced CKD may be the result of a compensatory protective response to kidney dysfunction, as an analogue to the elevated natriuretic peptide level during heart failure.\textsuperscript{[31]} The loss of thymosinβ4 from the circulation may be substantially higher in hemodialysis than in subjects with residual kidney function, which supports our postulation. Therefore, detailed studies comparing plasma and urinary/spent dialysate levels of thymosin β4 in CKD are warranted to assess the potential its impact, especially in hemodialysis.

Another observation is the enrichment of albumin fragments in urine of the CKD patients, but not in plasma or spent hemodialysate. This is expected since albuminuria is a frequent feature of CKD.\textsuperscript{[12]} When investigating the urine peptidomes from a population-based cohort,\textsuperscript{[25]} the albumin fragments were no longer abundant (Table 1B,D). By comparing the most abundant urinary peptide fragments between the two groups (Figure S1, Supporting Information), we observed that most of the top peptides identified in CKD patients (mostly albumin and α1-antitrypsin) were of low abundance in control subjects (Figure S1, Supporting Information, left). Albumin and α1-antitrypsin are plasma-derived and typically present only at a very low level in the urine of healthy individuals.\textsuperscript{[13]} In contrast, top peptides (mainly collagen type I and III, as well as fibrinogen α chain) identified from the population-based cohort showed similar relative abundance in CKD patients (Figure S1, Supporting Information, right). These data indicate that CKD may not affect the fragmentation of collagens and fibrinogens by proteases in the kidney.

In all three biofluids, collagens (mainly type I and III) and fibrinogens manifested high signal intensity. Based on the published data, collagens were proposed as biomarkers for diagnosis and prognosis early kidney and/or heart-related diseases,\textsuperscript{[25,34]} possibly indicating molecular changes in the extracellular matrix during fibrosis.\textsuperscript{[7,35]}

Our study has certain limitations. We could not obtain information about CKD etiologies of the HD patients, because their samples were collected anonymously. Association of peptides with CKD etiology was not the aim of the study, which is also not powered for this purpose. This study focuses on the inter-biofluid differences, we did not examine the possible intra-biofluid differences induced by medical treatment. Furthermore, the sample size is insufficient to assess the impact of medication, given the heterogeneity of the disease and the cohort, and the impact of multiple and diverse drugs applied in the patients. However, this does not have a major impact on the consistency of the results as the samples were matched in sex and age.

We are aware that the comparison between plasma and spent hemodialysate samples from the same HD patients would be most appropriate. Because of the lack of plasma samples from HD patients, we used plasma samples from advanced-stage CKD patients for consistency. The spent hemodialysate from HD patients may not be fully comparable to results obtained from CKD patients and healthy subjects. However, it is not possible to perform hemodialysis on individuals with preserved kidney function, due to evident ethical and medical reasons. We are aware that our current study is exploratory; the inclusion of more
samples, particularly from the HD patients, would be a logical next step to refine the existing results.

Another limitation is the moderate percentage of sequenced peptides (roughly 20% across the three body fluids). A major cause for this shortcoming appears to be the presence of unknown PTMs, prohibiting the correct matching of the spectra with the proteome database.\textsuperscript{[36]} Frequently, good spectrum quality still does not enable the assignment of sequence. Among others, fragment signals typically for glycosylation are present in many of the spectra where no sequence could be assigned.\textsuperscript{[1,37]}

As a result, we are not aiming at improving peptide sequencing by investigating potential posttranslational modifications in more detail.

The gold standards to assess kidney function are urinary albumin level and eGFR, a derivative based on serum creatinine levels.\textsuperscript{[38]} Both of them are reliable biomarkers in determining the disease severity and therefore of substantial value in providing clinical guidance. However, both serum creatinine and albuminuria have shortcomings as biomarkers for early detection or guiding intervention in CKD. The elevation of serum creatinine is not conspicuous until a substantial fraction of renal function is lost. The loss can be as huge as 50%, likely due to the accompanying reduction in muscle mass as CKD progresses.\textsuperscript{[39]} As a result, the diagnosis of CKD based on eGFR is generally too late for effective intervention. Urinary albumin excretion has been proposed as a better predictor of accelerated renal function decline than eGFR.\textsuperscript{[40]} However, it is highly variable\textsuperscript{[41]} and lacks accuracy in assessing renal function decline: neither the presence nor the absence of albuminuria can detect or preclude CKD with certainty.\textsuperscript{[42]}

Despite the technological advances in clinical proteomics, to date, only a limited number of biomarkers based on MS are in use. The reason for this gap does not appear to be technical (e.g., sample handling or storage), but mostly a result of a lack of appropriate studies. Most of the MS-based biomarker studies focus on preliminary discovery in very small cohorts, but not on the biomarker validation/qualification, due to the significantly larger effort involved. As a result, the biomarkers are typically not brought forward to be employed in patient assessment.\textsuperscript{[41]}

A change in attitude toward appropriately powered studies aiming at the validation of biomarkers has been proposed, which appears to be the solution to the limitations of existing biomarkers in CKD.\textsuperscript{[44]}

In summary, our study reports an in-depth characterization of three different peptidomes using the same MS platform. Comparing urine, plasma, and spent hemodialyseate is expected to widen our understanding of the underlying molecular differences associated with endogenous peptide processing. Furthermore, this study reveals several interesting points: a) significant positive correlations between urine and plasma with HD fluid and no correlation between urine and plasma based on the overlapping peptide abundance, indicating selectivity of the tubular reabsorption via a yet unknown molecular mechanism; b) identification of thymosin $\beta_4$ as highly abundant in the HD fluid; and c) a significant negative correlation of urinary thymosin $\beta_4$ with eGFR. Future experimental studies are warranted to explore the biological relevance of thymosin $\beta_4$ in the context of CKD.
Data Availability Statement
The mass-spectrometry proteomics data were deposited to the ProteomeXchange Consortium with an identification number 421225.

Conflict of Interest
T.H. and M.P. are employed by Mosaiques Diagnostics GmbH (MOS); H.M. is the co-founder of MOS; other authors declare no conflict of interest.

Keywords
hemodialysis fluid, peptidomes, plasma, urine

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