Quantitative protein mass spectrometry for kidney injury biomarker translation towards the clinical laboratory

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Chapter 5

Multiplex LC-MS/MS testing for early detection of kidney injury: a next-generation alternative to conventional immunoassays?

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Abstract

Background

LC-MS/MS has enabled the translation of many novel biomarkers to the clinical laboratory, but its potential for measurement of urinary proteins is still unexplored. In this study we examined the correlation and agreement between immunoassay and LC-MS/MS in the quantitation of kidney injury biomarkers and evaluated the application of technical LC-MS/MS meta-data assessment to ensure test result validity.

Methods

NGAL, IGFBP7, TIMP2, and KIM-1 were quantified in 345 urine samples with one multiplex lab-developed test that combines immunocapture with mass spectrometry read-out and 4 singleplex sandwich-type immunoassays. Assay performance and imprecision were monitored by 2 urine-based quality controls. Ion ratios, signal intensity, and retention time were monitored over all study samples.

Results

The LC-MS/MS retention time drift was ≤1.2%, ion ratios were within 20% of the target values at concentrations of >100 pmol/L, and peptides originating from the same protein were in agreement (slopes between 1.03 and 1.41). The interassay CV was between 9.3% and 19.1% for LC-MS/MS analysis and between 4.2% and 10.9% for immunoassay. Direct LC-MS/MS analysis was correlated with immunoassay in the quantitation of NGAL ($r = 0.93$; range: 0.01-37 nmol/L), IGFBP7 ($r = 0.80$; range: 0.01-2.6 nmol/L), TIMP2 ($r = 0.85$; range: 0.01-6.3 nmol/L), and KIM-1 ($r = 0.70$; range 0.01-0.4 nmol/L), but the analytical methodologies differed in measurands and calibration strategies.

Conclusions

LC-MS/MS is explored as a next-generation technology for multiplex urinary protein measurement. It has great potential to overcome nonselectivity and lack of standardization because of its capability of directly measuring well-defined molecular proteins.

Introduction

LC-MS/MS has been proposed as a next generation analytical strategy to substitute immunoassay (IA)-based technologies for protein biomarker quantitation in complex biological matrices. [1] The main benefits of LC-MS/MS are analytical selectivity, unequivocal characterization and detection of the measurand, multiplexing capability, and the use of embedded technical meta-data (e.g., ion ratio, internal standard monitoring) and interpeptide agreement evaluation in the postanalytical phase to validate test results. [2, 3] Previously, we used LC-MS/MS to quantify multiple proteins in complex biological matrices to target unmet clinical needs in kidney injury diagnosis by analyzing urinary NGAL, IGFBP7, TIMP2, and KIM-1. [4] Understanding the degree of correlation and discordances of direct and indirect measurements of these urinary proteins made by both LC-MS/MS and IA enables comparison between studies and laboratories using these methods.

The application of IA or LC-MS/MS for protein quantitation each have particular benefits and drawbacks. IA is known for its high precision, robustness, and suitability for automation for time-efficient analysis but is more prone to specificity errors, especially when multiplexing low-abundance proteins. [5, 6] Moreover, the selectivity of the measurand in IA principally relies on the antibodies used, since optical or chemiluminescence read-out systems indirectly detect the protein measurand. In contrast, the analytical selectivity of LC-MS/MS is achieved by direct detection of the mass:charge ratio (*m/z*) of the proteotypic peptide measurand(s). Moreover, an additional level of selectivity in quantitative protein mass spectometry (MS) may be achieved through evaluation of interpeptide agreement during the postanalytical assessment. Therefore, LC-MS/MS–based protein analysis strategies may reveal IA selectivity flaws that would otherwise remain unnoticed. [6] However, protein quantitation by LC-MS/MS is technically complex and has multiple error-sensitive sample preparation steps and a time-consuming preanalytical phase.

TIMP2, KIM-1, NGAL, and IGFBP7 are low-abundance proteins, present in picomolar to nanomolar concentrations, and demand a sensitive and selective analytical platform for quantitation. To date, concentrations of these biomarkers can be determined by either lab-developed tests (LDTs), research use–only sandwich ELISAs, or CE-marked IA-based tests. [7, 8] We developed a LDT for multiplex NGAL, IGFBP7, TIMP2, and KIM-1 quantitation to facilitate parallel biomarker evaluation and translation towards the clinical laboratory. [9] This LDT combines immunocapture with an LC-MS/MS read-out to achieve the desired level of analytical sensitivity and selectivity. In this study, we aim to determine the correlation and agreement between the multiplex LDT and 4 singleplex ELISAs in the quantitation of NGAL, IGFBP7, TIMP2, and KIM-1 from urine samples of

renal allograft donors and recipients. By exploring method transferability and LC-MS/MS data validity, we aim to unveil the opportunities and limitations of multiplex kidney injury biomarker analysis by LC-MS/MS.

Material and methods

To cover the expected wide urinary concentration range of TIMP2, KIM-1, NGAL, and IGFBP7, 343 deidentified urine samples were selected from the REnal Protection Against Ischemia-Reperfusion in transplantation (REPAIR) clinical trial. [10] Ethical approval for the study in the United Kingdom was given by the Joint University College London/University College London Hospital Committees on the Ethics of Human Research in June 2009 (reference number 09/H0715/48). In the Netherlands, the trial was approved by the central Medical Ethical Committee. Between January 2010 and April 2013, spot urine samples were collected and centrifuged at 400 *g* for 10 min and the supernatant was stored at -80°C until analysis. Samples underwent 2 and 3 freeze–thaw cycles before ELISA and LC-MS/MS analysis, respectively.

Urinary proteins were quantified in singleplex by 4 research-use only ELISA kits: NGAL (R&D Systems), IGFBP7 (BOSTER Biological Technology), TIMP2 (Quantikine, BioTechne), and KIM-1 (R&D Systems) according to the manufacturer's instructions. The kits included calibrators based on recombinant protein. Detailed information about the reagents and analytical procedures can be found in the online supplementary information (Supplemental methods).

Proteins were quantified in multiplex in a total of five LC-MS/MS analysis batches by the LDT. [9] In each batch, 2 urine-based internal quality controls and 5 urine-based external calibrators were prepared and analyzed together with the samples. For NGAL, TIMP-2, and KIM-1, calibrators were value-assigned in pmol/L by recombinant protein spiking, whereas IGFBP7 was value-assigned by ELISA.

ELISA results were converted to molar units using the molecular weight based on the canonical amino acid sequence in the UniProt database. [11] Mass Hunter Workstation software, version 10.0 (Agilent Technologies), was used for LC-MS/MS peak integration. Statistical analysis and graphics were generated in R (version 4.0.2). Passing-Bablok regression with Spearman rank correlation coefficient are provided.

Results

NGAL, IGFBP7, TIMP2, and KIM-1 were quantified by IA and LC-MS/MS, both using antibody-based enrichment but different read-out technology, in 343 urine samples from kidney transplant donors and recipients and were found to be present in the picomolar to nanomolar range (Table 1). IGFBP7, TIMP2, and NGAL were detected by LC-MS/MS in >93% of urine specimens, whereas KIM-1 could be detected in 61% of the samples (limit of detection ±1 pmol/L). IA analysis for NGAL, IGFBP7, TIMP2, and KIM-1 provided results for all urine samples (all >5 pmol/L). The interassay analytical imprecision of 10 LC-MS/MS measurements over 5 days was between 9.3% to 25.0%, depending on the concentration and peptide, while the interassay imprecision by ELISA was 3.6% at 172 pmol/L and 4.2% at 244 pmol/L (n = 15) for TIMP2 and 9.4% at 757 pmol/L and 10.9% at 2053 pmol/L for IGFBP7 (n = 20) (Table 1). The mean intraassay of KIM-1 and NGAL ELISAs was obtained by calculating the percentage difference between duplicates in the calibration curves and were 5.9% and 7.3%, respectively.

LC-MS/MS analysis enables postanalytical data validity assessment for clinical samples utilizing embedded technical metadata, such as measurand retention time, ion ratio, and internal standard signal intensity. The robustness of liquid chromatography performance, specified by retention time drift over all urine samples, ranged from CV = 0.66% to 1.21%. The analytical specificity was assessed by ion ratio monitoring, which is the ratio of qualifying product ion peak area over the quantifying product ion peak area. Ion ratios deviate ≤20% from the target at concentrations >100 pmol/L (Supplemental Fig. 1). An equal amount of internal standard was added to all samples to correct for variances introduced in the (pre)analytical phase; internal standard peak areas were monitored in clinical samples to identify outliers and/or matrix effects. Two samples were excluded based on internal standard signal (peak area cutoff ≤2000 counts) (Supplemental Fig. 2). Finally, the validity of the results was assessed through interpeptide comparisons for peptides originating from the same protein. For NGAL, IGFBP7, and TIMP2, results were in agreement, but the qualifying peptide of KIM-1 performed less well, yielding only 35 results eligible for comparison (Supplemental Fig. 3).

Concentrations obtained by LC-MS/MS and IA were compared and correlation coefficients and slopes for plots comparing the paired measurements were r = 0.926, slope = 2.41 (95% CI: 2.31, 2.70) for NGAL; r = 0.800, slope = 1.08 (95% CI: 0.95, 1.20) for IGFBP7; r = 0.846, slope = 0.43 (95% CI: 0.38, 0.47) for TIMP2; and r = 0.697, slope = 0.42 (95% CI: 0.38, 0.49) for KIM-1 (Figure 1). Deviations from slope = 1 are largely due to the calibrators used for converting results into concentrations in molar or mass units, while lower r values may indicate variations in the measurand or lower measurement precision.

The LC-MS/MS calibrator of IGFBP7 was value-assigned by the ELISA kit, resulting in a slope closer to 1 compared to the other 3 proteins, for which different calibrator strategies were used. The poor correlation and data scatter at concentrations <100 pmol/L for all biomarkers are likely due to increased measurement uncertainty. This is supported by unstable ion ratios observed at concentrations below approximately 100 pmol/L in LC-MS/MS analysis.

Table 1: Imprecision for an in-house developed LC-MS/MS versus RUO ELISA.

Biomarker	LC-MS/MS			ELISA		
	Measuring range (pmol/L)	Inter-day imprecision		Inter-day imprecision		Intra-day imprecision
		IQC1	IQC2	IQC1	IQC2	Cal curve
		%CV	%CV	%CV	%CV	%CV
NGAL	10 - 36,875	14.3	17.4	*	*	7.3
IGFBP7	10-2,615	13.0	25.0	9.4	10.9	**
TIMP2	10 - 6,285	10.9	9.3	3.6	4.2	**
KIM-1	10 - 355	19.1	16.4	*	*	5.9

*Inter-assay imprecision data of IQC samples not available; ** Not applicable, QC data available.

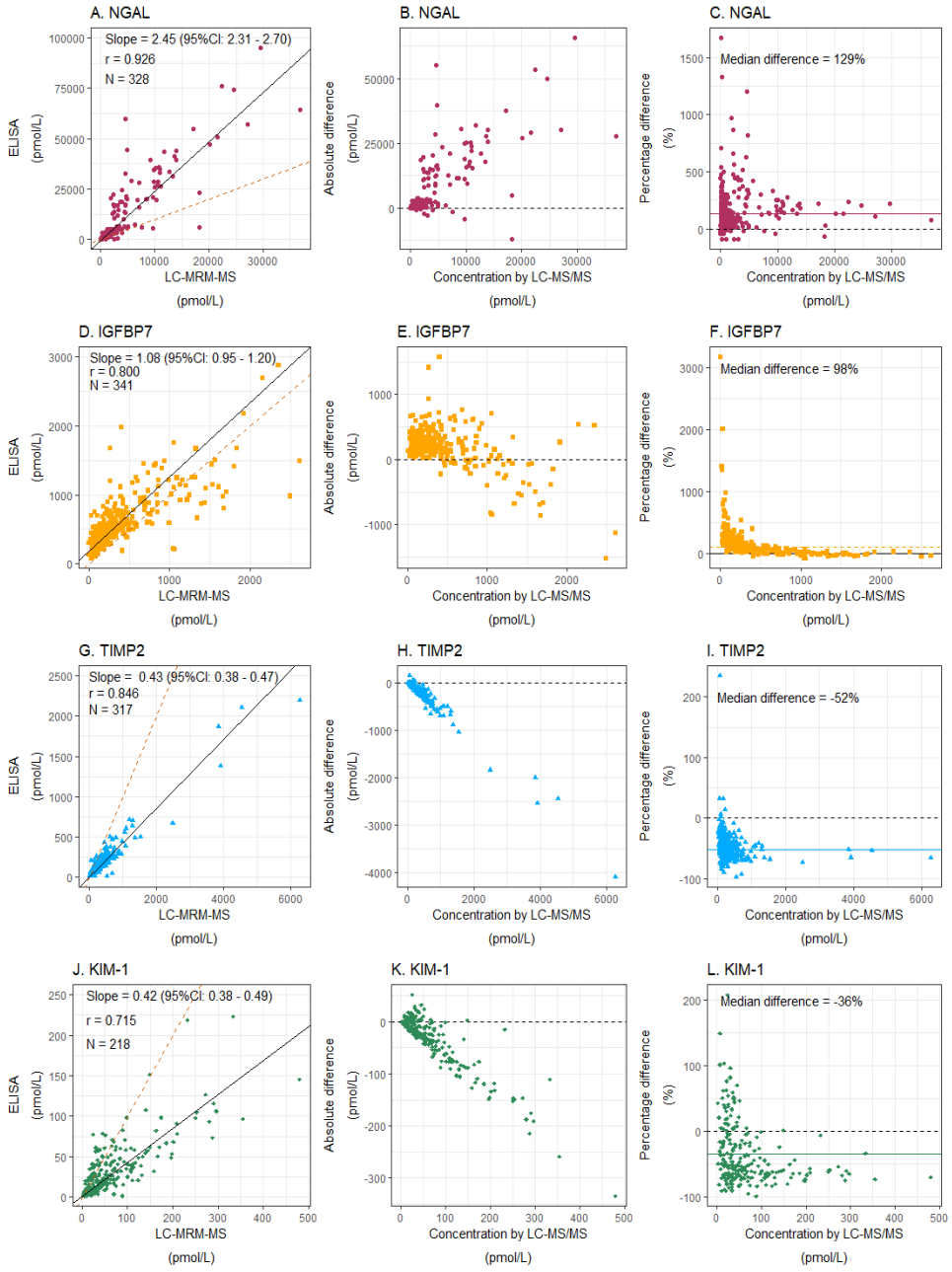

Figure 1. Measurement procedure comparison of multiplex LC-MS/MS and single plex research-use only ELISA kits for the quantitation of NGAL, IGFBP7, TIMP2 and KIM-1 from urine. Left column: Passing-Bablok regression (solid line) and line of identity (red dashed). Middle column: Difference plots, showing absolute differences between the paired measurements plotted against the results obtained by LC-MS/MS. Right column: Difference plots, showing percentage differences between the paired measurements plotted against the results obtained by LC-MS/MS, with the median percentage difference represented by solid lines.

Discussion

To explore the value and analytical performance of LC-MS/MS in biomarker translation, we made use of LC-MS/MS metadata for quality assessment and present a head-to-head comparison of IA-based ELISAs and MS-based test results for translating promising urinary biomarkers from bench to bedside. While LC-MS/MS allows confirmation of peptide (and hence protein) identities through ion ratio monitoring, the spectrophotometric detection of ELISAs does not; instead, in IAs whole protein complexes are measured and therefore, by design, analytically less selective than LC-MS/MS. However, it is important to note that the MS-based application described here is still in its exploratory phase. While it is sufficiently developed for use as a second-tier test, [12] its imprecision and turnaround time cannot match the performance of commercially available ELISAs.

Calibration biases were observed for these urinary biomarkers in this method comparison, which can be explained by different (recombinant protein-based) calibration strategies and the lack of international recognized reference materials. The nonequivalence of test results between the IA- and MS-based analytical strategies emphasizes the need for test standardization from the initial development to increase transferability of results. Correspondingly, Ji et al. reported moderate NGAL test agreement with a slope of 0.71 (95% CI: -0.67, 0.77) between a particle-enhanced turbidimetric IA and an LC-MS/MS-based LDT. [13]

Beyond calibration bias, the correlation between direct and indirect IA-based proteins tests was suboptimal. This sample-specific bias may be explained by differences in measurand and measurement uncertainty, which was relatively larger in our LC-MS/MS analyses. In IA, a mixture of different proteoforms from a single protein may be measured and the recovery is a function of theantibody types that have been used. The LC-MS/MS test, on the other hand, combines immunocapture, using polyclonal antibodies with variable epitope specificities, with a peptide-specific detection method. Importantly, antibody affinity and avidity are influenced by tertiary and quaternary protein structures, and while the kidney injury biomarker proteins are assumed to be soluble and freely present in urine, they are often part of protein complexes. Specifically, only the soluble cleaved ectodomain of transmembrane protein KIM-1 is quantified by IA. [14] Furthermore, NGAL may be present in urine as monomer, homodimer, and heterodimer with MMP9, [15] while TIMP2 is detected in complex with its active substrate MMP9, and IGFBP7 is typically bound to insulin and its growth factors. [16] The molecular presentation of the proteins of interest affect their affinities for the antibodies used in IAs, potentially leading to variable results. [15]

Strengths of this study are the unveiling of both calibration and sample specific bias in LC-MS/MS test results compared to IA. In addition, MS-based tests will, by design, allow adequate characterization of calibrator(s) and selective measurement of potentially clinically relevant proteoforms in biological specimens.

There are limitations to this study that need to be acknowledged. First, the LC-MS/MS methodology was not compared with commercially available regulatory approved tests and therefore could only be considered for research use only. Because the current LC-MS/MS test can be considered as a second-tier test, it is too preliminary to compare the methodology to commercially available CE-marked medical tests. If the current LC-MS/MS test would be developed into a first-tier diagnostic test, comparison to commercially available tests is needed. Second, internal quality controls were used to investigate the between-day variances of NGAL, IGFBP7, TIMP2, and KIM-1, but imprecision data were incomplete for ELISA-based KIM-1 and NGAL measurements. Third, the urine specimens were stored long-term, up to 10 years at -80°C, prior to analysis, and an additional freeze–thaw cycle was required for LC-MS/MS analyses. Urine biomarker instability and/or degradation and unfolding is an important determinant of the preanalytical phase, which affects biomarker recovery. Besides the measurand, the analytical platform used in the analytical phase determines the susceptibility to biomarker recovery. Moreover, the epitope accessibility and integrity determine the antibody-target affinity and resident time and protein recovery. The interplay between measurand intactness and technology can affect biomarker recovery. Previous studies evaluating biomarker stability were generally performed using IA. For instance, Pennemans et al. studied urinary KIM-1 stability and stated that freeze–thaw cycles should to be avoided, [17] whereas Schuh et al. reported a decrease of <3% in KIM-1 and NGAL concentrations after 3 consecutive freeze–thaw cycles. [18] In our hands, protein recovery by LC-MS/MS was considered stable for up to 4 freeze–thaw cycles (Supplemental Table 1).

In conclusion, we demonstrate that LC-MS/MS has clear potential as a next-generation measurement platform for absolute quantitation of urinary proteins. The advantages it offers over IAs are its analytical selectivity, a molecular definition of the measurands, and its potential for ab initio medical test standardization. Moreover, the versatility and multiplexing capability of MS facilitates efficient translational biomarker research and the development of in-house tests based on clinical needs. Finally, LC-MS/MS has the potential to capture biological complexity for future precision diagnostics.

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Supplementary data chapter 5

Supplementary Information I: Methods

Materials and reagents

Formic acid (FA, ≥ 99% purity) was purchased from VWR (Radnor, PA, USA) and LC-MS grade methanol (MeOH) was purchased from Biosolve (Valkenswaard, The Netherlands). Iodoacetamide (IAM), and ammonium bicarbonate (ABC) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and Tris (2-carboxyethyl)phosphine (TCEP) was purchased from Thermofisher Scientific (Waltham, MA). ELISA kits for TIMP2 (Cat. Nr. DTM200, Quantikine, BioTechne, Minneapolis, MN, USA), IGFBP7 (Cat. Nr. EK0991, BOSTER Biological Technology, Pleasantion, CA, USA), KIM-1 (Cat. Nr. DY1750, R&D Systems, Minneapolis, MN, USA) and NGAL (Cat. Nr. DY1757, R&D Systems, Minneapolis, MN, USA) quantitation were purchased from their respective manufacturers. Nunc Maxisorp™ plates for ELISA development and streptavidin-coated magnetic beads (Cat. Nr. 65601, T1 DynaBeads™, MyOne™) for antibody immobilization prior to LC-MS/MS were purchased from InvitrogenTM (ThermoFisher Scientific, Waltham, MA). Biotinylated antibodies against human TIMP2 (Cat. Nr. BAF971), IGFBP7 (Cat. Nr. BAF1334), KIM-1 (Cat. Nr. BAF1750) and NGAL (Cat. Nr. BAF1757) were purchased from R&D systems (Minneapolis, MN, USA). Recombinant TIMP2 (Cat. Nr. NBP2-22869) and NGAL (Cat. Nr. NBP1-50987) were from Novus Biologicals (Centennial, CO, USA) and recombinant KIM-1 (Cat. Nr. LS-G97633) was purchased from LS Biologicals (Seattle, WA, USA). Sequencing-grade porcine modified trypsin was purchased from Promega (Cat. Nr. V5111, lot: 0000430387, Madison, WI, USA). Synthetic peptides, unlabeled and stable-isotope labeled (SIL)([$^{13}C_6$, $^{15}N_2$]-lysine or [$^{13}C_6$, $^{15}N_4$]-arginine) were synthesized in-house.

Immunoassay analysis

In the immunoassays used in this study, monoclonal anti-human (h) KIM-1 raised in mouse, monoclonal anti-hTIMP2 (host manufacturer proprietary), anti-hNGAL from rat (clonality manufacturer proprietary) and polyclonal anti-hIGFBP7 from goat were used as capture antibodies. HRP-conjugated polyclonal anti-hTIMP2 (host unknown), biotinylated polyclonal goat anti-hKIM-1, biotinylated goat anti-hNGAL (clonality manufacturer proprietary) and biotinylated polyclonal goat anti-hIGFBP7 were used as the detection antibodies. The antibodies for capture of human KIM-1 and NGAL were coated on Nunc Maxisorp plates by overnight incubation at 4°C. After antibody coating, plates were washed three times with 0.05% (v/v) Tween20 in PBS, blocked with 1% (w/v) BSA/0.05% (v/v) Tween20 in PBS at RT for one hour and washed three times with 0.05% (v/v) Tween20 in PBS. Samples (urine samples, standards, controls or in-house developed QC samples) were incubated with the plate-coated capture antibodies at RT for 120 minutes (for TIMP2, NGAL and KIM-1) or at 37°C for 90 minutes (for IGFBP7).

After 3-4 wash steps with 0.05% (v/v) Tween20, samples were incubated with biotinylated detection antibodies at RT for 120 minutes for TIMP2, NGAL and KIM-1 and at 37°C for 60 minutes for IGFBP7. Afterwards plates were washed with 0.05% (v/v) Tween(R)20, or if provided, the wash buffer for the ELISA kits, and samples were incubated with streptavidin or avidin with horseradish peroxidase (HRP) conjugate in the dark at RT for 30 minutes (for NGAL, KIM-1) or at 37°C for 30 minutes (for IGFBP7) and washed with 0.05% (v/v) Tween-PBS.

The samples were then incubated with chromogenic substrate 3,3'5,5'-tetramethylbenzidine (TMB) at RT in the dark for 20-30 minutes before the enzymatic oxidation reaction was quenched with 0.5 or 1 M sulfuric acid (H_2SO_4) and absorbance was read with a microplate reader at 450 nm. To convert absorbance readings into concentrations, standard curves with recombinant human TIMP2, NGAL, IGFBP7 and KIM-1 were prepared according to ELISA manufacturer instructions.

LC-MS/MS analysis

The kidney injury biomarkers NGAL, IGFBP7, TIMP2 and KIM-1 were measured in urine samples using a validated multiplex LC-MS/MS method.(9) In short, immunocapture was performed using polyclonal and biotinylated anti-NGAL, anti-hIGFBP7, anti-hTIMP2 and anti-hKIM-1 coupled on streptavidin-coated magnetic beads. Freshly mixed bead suspension was added to thawed 50 µL urine sample and incubated at RT for 120 min. After immunocapture, 20 µL of a 1.5 nM stable-isotope labelled (SIL) peptide mixture was added as internal standard (IS), proteins were denatured and disulfide bonds reduced with 5 mM TCEP and cysteine residues alkylated with 5 mM IAM for stabilization. Proteins were digested into peptides with 0.4 µg trypsin at 37°C for 18 hours prior to LC-MS/MS analysis. Samples were analyzed on a 1290 UPLC system, with a Zorbax SB-C18 with 1.8 µm particle guard column (2.1 x 5 mm) and analytical column (2.1 x 50 mm), coupled to a triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) performing in dynamic multiple reaction monitoring (dMRM) mode. Peptides were separated at a flow of 0.3 mL/min, with 1% (v/v) MeOH/0.05% (v/v) FA in MQ water and 95% (v/v) MeOH/0.05% (v/v) FA in MQ as mobile phases, using a nonlinear gradient optimized for dynamic MRM and time-efficient analysis. The total run time per sample was 14 min including 3 min post-time for column equilibration. The LC-MS/MS instrument performance was considered ccepatble if the following criteria were met: peptide relative response (RR) CV <10% for each peptide, retention time drift ≤ 0.2 minutes and ion ratio CV <20% over five injections of a system suitability sample prior to and after study sample measurement.

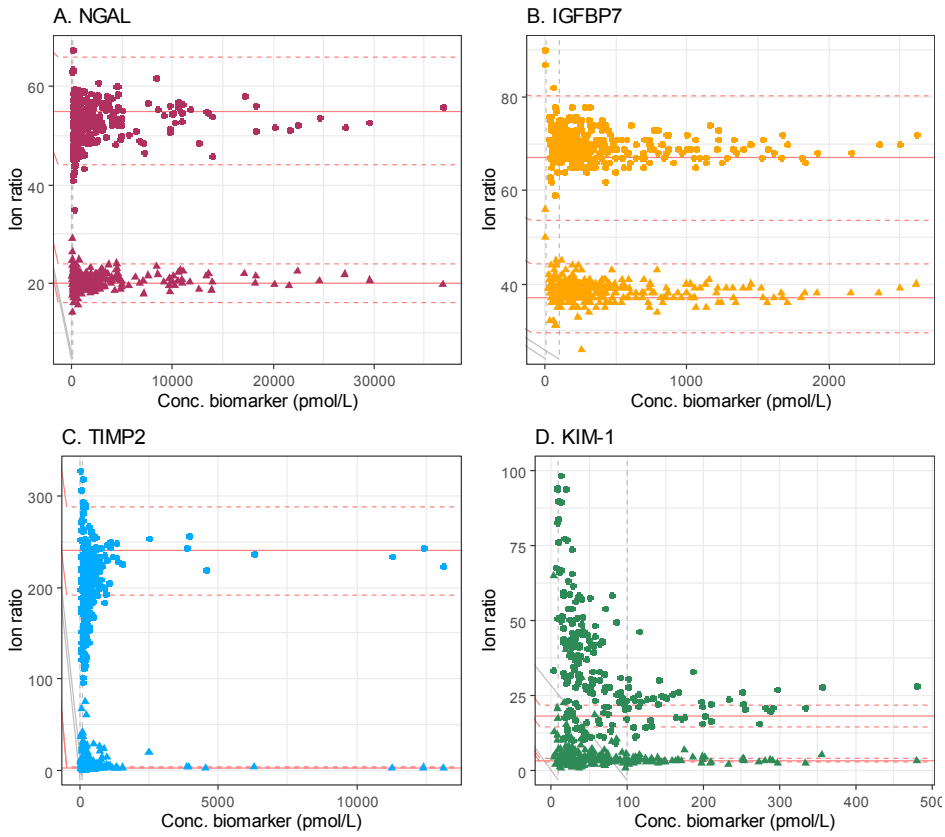


Figure S1. Ion ratio monitoring in urine samples. For each peptide, one precursor ion and three collision-induced dissociation-generated product ions were monitored by multiple reaction monitoring (MRM) analysis. One product ion was used for quantitation and two product ions were monitored for qualification to confirm the results. The ion ratio is the ratio of qualifying product ion peak area over the quantifying product ion peak area and provides information about accuracy of MS analysis. The ion ratio of the first qualifier ion (▪) and second qualifier ion (Δ) are shown with the target value, set at method validation, represented by the solid line and the 20% error boundaries represented by the horizontal dashed lines. The vertical dashed lines are set at 10 pmol/L (initially set lower limit of quantitation, LLoQ) and 100 pmol/L (based on ion ratios). For TIMP2 (C), the poor peptide selectivity of the qualifying ion y^{3+} (m/z = 275.2) can be recognized by the inconsistency in ion ratio.

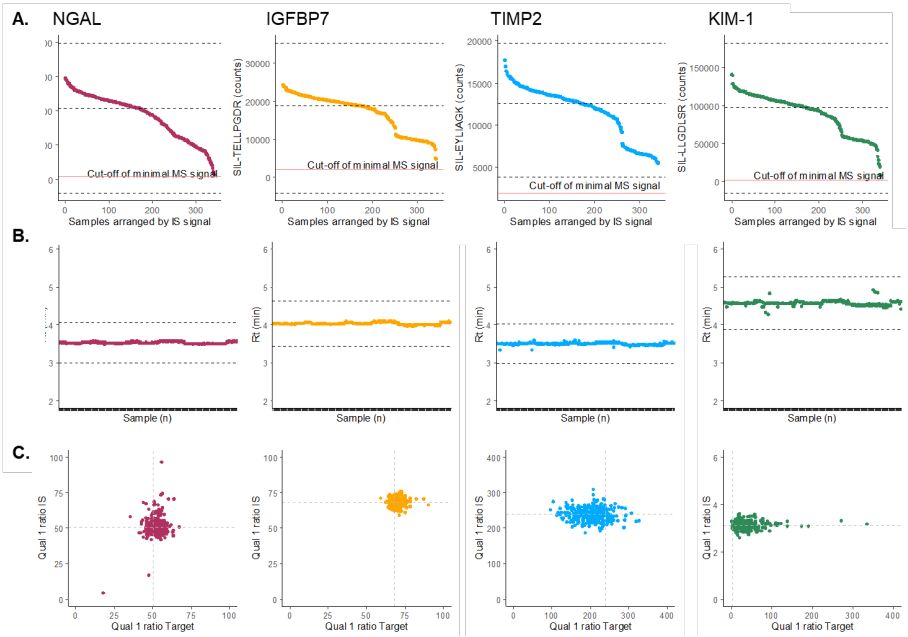
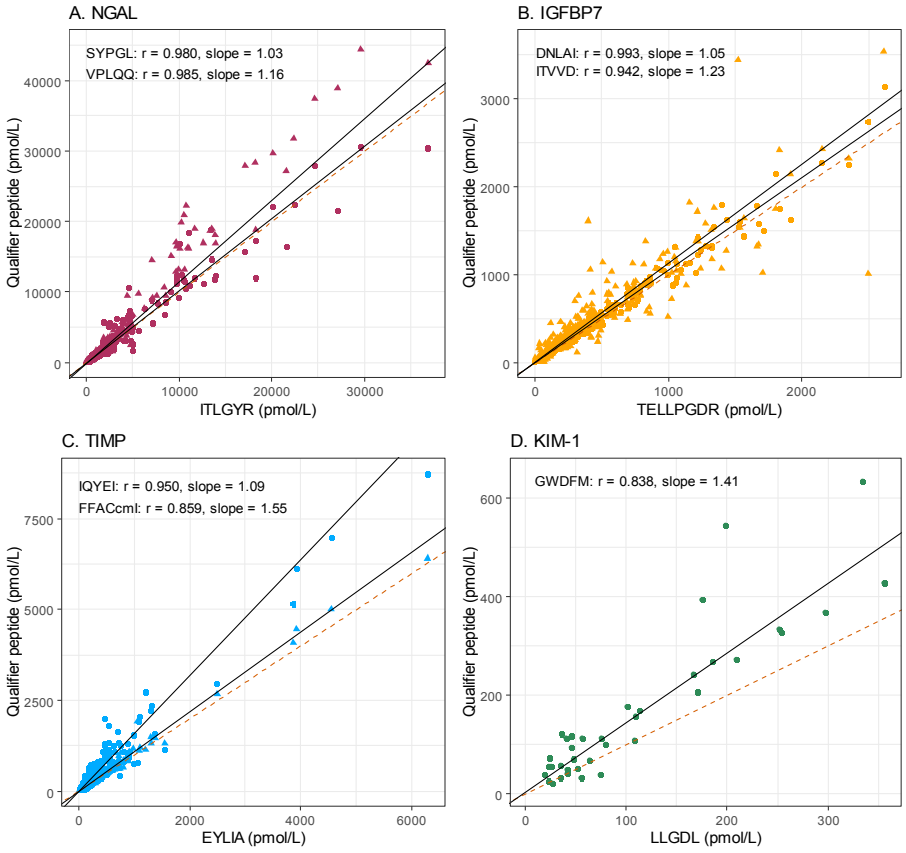


Figure S2: LC-MS/MS data validity assessment. Study samples were arranged by signal intensity of the internal standard (IS). Samples with an IS peak area below 2000 were considered invalid and excluded for analysis (n = 2) (A). Peptide retention time was monitored to examine LC stability over time. (B). The ion ratio of the target peptide plotted against the ion ratio of the IS to identify outliers due to interferences in LC-MRM-MS analysis or peak integration flaws (C). Data points are colored by protein (NGAL = red, IGFBP7 = yellow, TIMP2 = blue and KIM-1 = green. Data shown of the quantifying peptides.



the quantifying peptide (x-axis) and the qualifying peptides (y-axis). The dashed line represents the line of identity. Peptide names are abbreviated by the first five amino acids. The slope in Passing-Bablok regression and the Spearman's rank correlation coefficient are shown.

Supplementary Information III: Stability evaluation

Six fresh spot urines were collected in urine containers without protease inhibitors (Screwcap, yellow lid, max. volume 100 mL, SARSTEDT) and aliquoted to study the effect of freeze-thaw cycle introduction (n = 6 samples) and pos-textraction stability. To study the effect of urine sample freezing on biomarker analysis, samples underwent 1 (standard protocol), 2 or 4 consecutive freeze-thaw cycles (-80 °C) and all samples were analyzed in one MS analysis batch. The post-extraction stability was examined by preparing a pooled urine sample for LC-MS/MS analysis and injecting 10 µL sample 1, 3, 6, 9, 12 , 18 and 24 hours and 7 days after extraction and stored in the LC system autosampler (4-8°C). The first injection, 1 hour after the addition of quench solution, was set as reference condition.

The stability results of the post-extraction spiking experiment are shown in Supplementary Table 1 and the protein stability after the introduction of consecutive freeze-taw cycles are shown in Supplementary Table 2.

Supplementary Table 1. Post-extraction stability at 4-8°C

Biomarker	Conc. (pmol/L)	Percental difference (%PD)							y-intercep (95%CI)	slope (95% CI)	p-value
	1 h (nominal)	3 h	6 h	9 h	12 h	18 h	24 h	7 days			
NGAL	806	-4.3	-0.5	-2.9	-8.1	5.4	-11	-3.4	783 (743 - 822)	0.21 (-0.53 - 0.46)	0.874
IGFBP7	407	-1.1	0.3	-13.2	-6.8	-5.2	-9.4	3.7	385 (364 - 406)	0.11 (-0.05 - 0.47)	0.096
TIMP2	1373	-1.1	5.8	-8.0	-12.3	-7.3	-4.1	-7.8	1326 (1248 – 1405)	0.41 (-1.37 - 0.59)	0.382
KIM-1	590	1.8	-3.5	-1.8	-3.8	-3.8	0.8	-4.0	582 (570 - 594)	0.06 (-0.25 - 0.06)	0.184

*Slope with 95% confidence interval and p-value from linear regression are given.

Supplementary Table 2. Freeze-thaw cycles and biomarker recovery

		1x freeze-thaw		2x freeze-thaw			4x freeze-thaw		
	IGFBP7	Recovery	IGFBP7	Recovery	Paired t test	IGFBP7	Recovery	Paired t test
Donor	(pmol/L)	(%)	(pmol/L)	(%)	p-value	(pmol/L)	(%)	p-value
1	77	100	69	90		55	72	
2	204	100	152	75		125	61	
3	862	100	1019	118		1351	157	
4	246	100	195	79		256	104	
5	1485	100	1333	90		1387	93	
6	1309	100	1288	98		1288	98	
Mean recovery with respect to 1x ft:				90	0.908		98	0.627
	NGAL	Recovery	NGAL	Recovery	Paired t test	NGAL	Recovery	Paired t test
Donor	(pmol/L)	(%)	(pmol/L)	(%)	p-value	(pmol/L)	(%)	p-value
1	843	100	927	110		-	-	
2	321	100	242	75		250	78	
3	4514	100	3210	72		5237	116	
4	96	100	112	116		112	116	
5	653	100	193	29*		486	74	
6	-	-	-	-		-	-	
Mean recovery with respect to 1x ft:				80	0.246		96	0.924
	TIMP2	Recovery	TIMP2	Recovery	Paired t test	TIMP2	Recovery	Paired t test
Donor	(pmol/L)	(%)	(pmol/L)	(%)	p-value	(pmol/L)	(%)	p-value
1	93	100	103	110		63	67	
2	51	100	59	114		65	127	
3	512	100	478	93		463	90	
4	60	100	58	96		57	95	
5	401	100	229	57		324	81	
6	371	100	228	61		264	71	
Mean recovery with respect to 1x ft:				89	0.151		89	0.072
	KIM-1	Recovery	KIM-1	Recovery	Paired t test	KIM-1	Recovery	Paired t test
Donor	(pmol/L)	(%)	(pmol/L)	(%)	p-value	(pmol/L)	(%)	p-value
1	24	100	26	107		15	63	
2	10	100	9	83		10	95	
3	283	100	256	91		292	103	
4	24	100	22	91		13	54	
5	124	100	62	50		91	73	
6	-	-	-	-		-	-	
Mean recovery with respect to 1x ft:				84	0.206		78	0.271