

Quantification of novel urinary protein safety biomarkers for the detection of glomerular injury in clinical samples

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INTRODUCTION

Biomarkers of kidney tubular injury have been extensively studied and qualified for nonclinical safety assessment and have also been applied at the cohort level in healthy volunteers in early-phase clinical trials when there is an a priori suspicion of nephrotoxicity. In contrast, data on urinary biomarkers for drug-induced glomerular injury remain limited, particularly for the detection of subclinical injury prior to changes in estimated glomerular filtration rate (eGFR) and/or albuminuria.

To address this gap, the Translational Safety Biomarker Pipeline (TransBioLine) initiative, supported by the EU Innovative Medicines Initiative, aims to qualify novel biomarkers for drug-induced organ injury and to improve the mechanistic understanding of renal safety risks. A key objective is the identification and evaluation of glomerular-specific biomarkers and to demonstrate how a multi-marker panel, when combined with established tubular injury biomarkers, can provide pathomechanistic insight and enable detection of subclinical glomerular injury in the absence of changes in serum creatinine or progressive proteinuria.

In this context, a multiplex assay platform based on immunoaffinity enrichment coupled with mass spectrometry detection (immunoaffinity LC-MS/MS) was developed and validated for use in human urine. The panel included podocyte-derived glomerular biomarkers - nephrin (NPHS1), podocin (NPHS2), and podocalyxin (PODXL) - in addition to two tubular and two vascular protein biomarkers. Urine samples from healthy volunteers (n = 50) and patients with histopathologically confirmed glomerular injury (n = 48), including conditions such as lupus nephritis, were analyzed.

TUBULAR AND GLOMERULAR PROTEIN BIOMARKERS

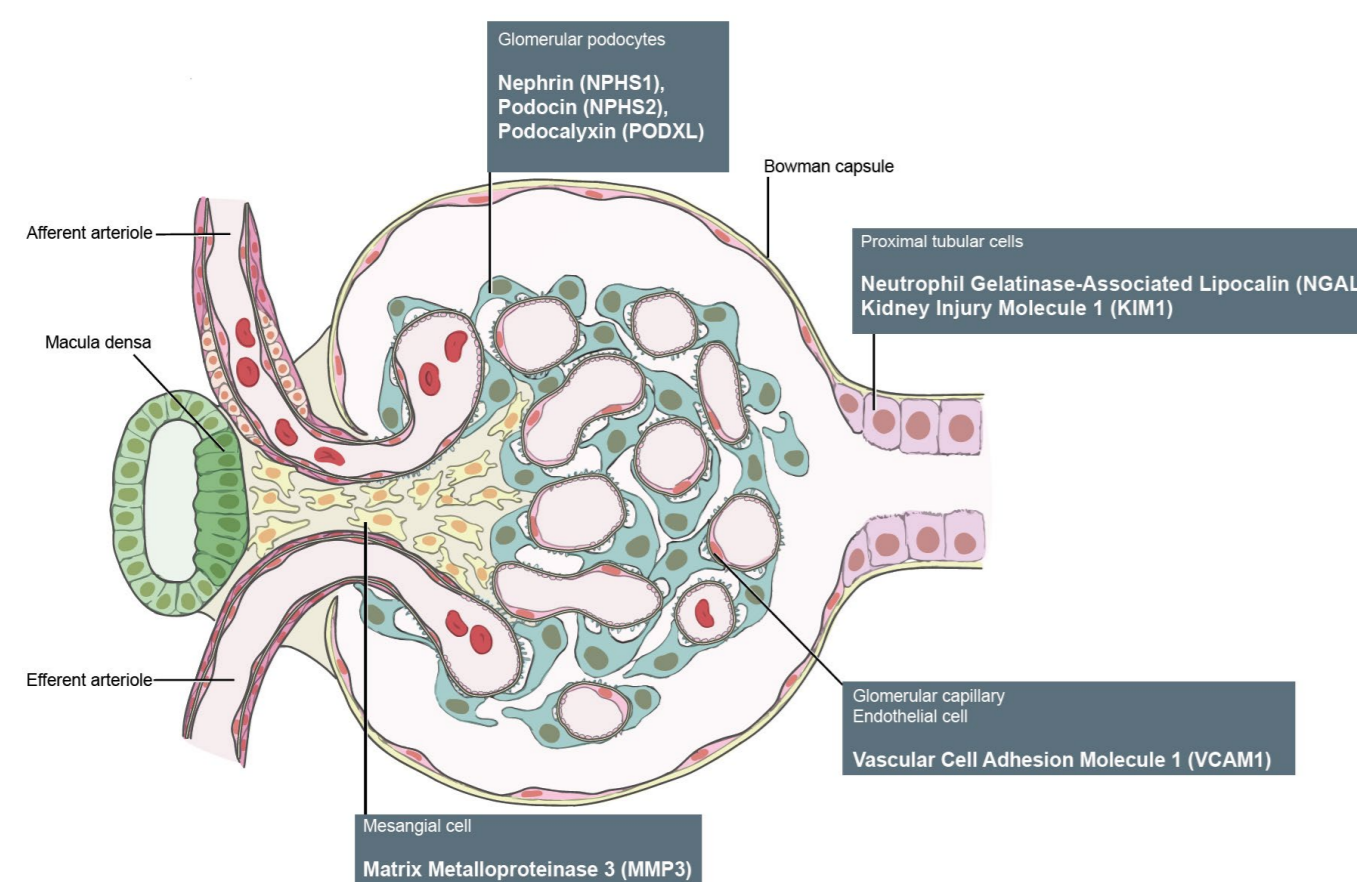


Figure 1: Nephritin (NPHS1), Podocin (NPHS2), Podocalyxin (PODXL) are glomerular injury biomarker candidates. Kidney Injury Molecule 1 (KIM1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) are tubular injury biomarkers. Vascular Cell Adhesion Molecule 1 (VCAM) and Matrix Metalloproteinase 3 (MMP3) represent proteins from the vascular compartment and are part of a biomarker panel pursued for the detection of vascular injury.

ACKNOWLEDGMENT

This work was funded by EU Innovative Medicine Initiative project TransBioLine (Project 821283).



METHOD - IMMUNOAFFINITY-LC-MS/MS

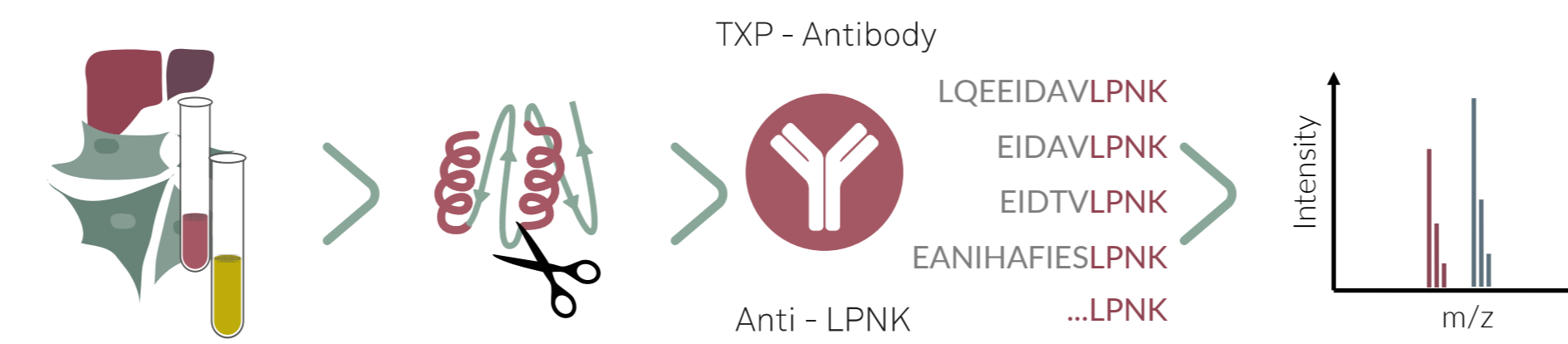


Figure 2: Immunoaffinity LC-MS/MS workflow. Urinary proteins are subjected to tryptic digestion, followed by the addition of isotopically labeled standard peptides. Group-specific antibodies targeting C-terminal sequence motifs are applied in an automated immunoprecipitation process to selectively enrich peptides derived from proteins of interest. Subsequently, unique biomarker-derived peptides are quantified by targeted nano LC-MS/MS using corresponding reference peptides for calibration.

A protein assay platform was established for the analysis of renal injury biomarkers in clinical urine specimens. Proteins were enzymatically digested using trypsin to generate peptides. Target peptides derived from the biomarkers were selectively enriched using multi-specific antibodies (TXP antibodies) directed against conserved C-terminal amino acid motifs present in the biomarker sequences, independent of species origin. The immunoaffinity-enriched peptides were subsequently detected and quantified by nano-liquid chromatography coupled to tandem mass spectrometry (immunoaffinity LC-MS/MS), using synthetic isotope-labeled standards for calibration and quantification.

ASSAY VALIDATION

Validation was performed in accordance with applicable FDA and EMA guidance documents. The following parameters were evaluated: reproducibility, parallelism, interference, accuracy and precision, and stability. Assessments were conducted using calibration standards, biological quality control (QC) samples, and patient samples. Representative data for inter-assay accuracy and precision in biological QC samples, as well as reproducibility for the candidate glomerular biomarker podocalyxin (PODXL), are provided in Tables 1 and 2. Accuracy and precision were evaluated by repeated analysis of biological QC samples (QC1, QC2, and QC3) across six independent analytical runs. QC samples were prepared by spiking urine from healthy donors with protein extracts derived from human kidney tissue.

Table 1: Inter-assay precision and accuracy was determined by repeated analysis of biological quality control samples (n=6). Acceptance criteria were as follows: Accuracy: 80%-120%; at LLOQ/ULOQ 75%-125%, Precision: $\pm 20\%$; at LLOQ/ULOQ $\pm 25\%$, & Total Error $\leq 40\%$

Run	QC1 ng/mL	QC2 ng/mL	QC3 ng/mL
Mean value, n= 6	102	25.3	9.37
SD	10.8	2.47	0.65
CV %	11	10	7
Nominal value	98.4	25.2	9.56
Accuracy %	104	101	98
TE %	14	10	9

Table 2: Reproducibility was tested in urinary samples from 15 donors (n=2). In at least 80% of the samples the percentage difference for results from two analytical runs should be within $\pm 30\%$. Difference (%) was calculated as follows: $100 \times (\text{result}_{\text{replicate 2}} - \text{result}_{\text{replicate 1}}) / \text{result}_{\text{average}}$

Run	Donor (mean value, n=2), ng/mL														
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
1	9.92	5.26	17.9	25.4	8.92	12.5	52.1	110	5.79	16.2	6.40	7.20	5.08	30.7	20.8
2	9.24	5.13	14.6	29.7	8.67	11.4	48.6	106	6.05	14.7	5.67	7.01	4.74	31.2	20.6
Difference %	-7	-2	-20	16	-3	-9	-7	-4	4	-9	-12	-3	-7	2	-1

RESULTS

Using IA-LC-MS/MS assays targeting urinary glomerular, tubular, and vascular proteins, we generated learning-phase data from single time-point samples collected from patients with histologically confirmed glomerular disease (n=50) and healthy controls (n=48). Preliminary analyses indicate that median biomarker levels were elevated in disease cases compared with controls for NGAL (5.7-fold), PODXL (2.1-fold), MMP3 (4.7-fold), and VCAM1 (5.9-fold). In contrast, the differences for NPHS1 (1.5-fold) and NPHS2 (1.5-fold) were less pronounced (Figure 3).

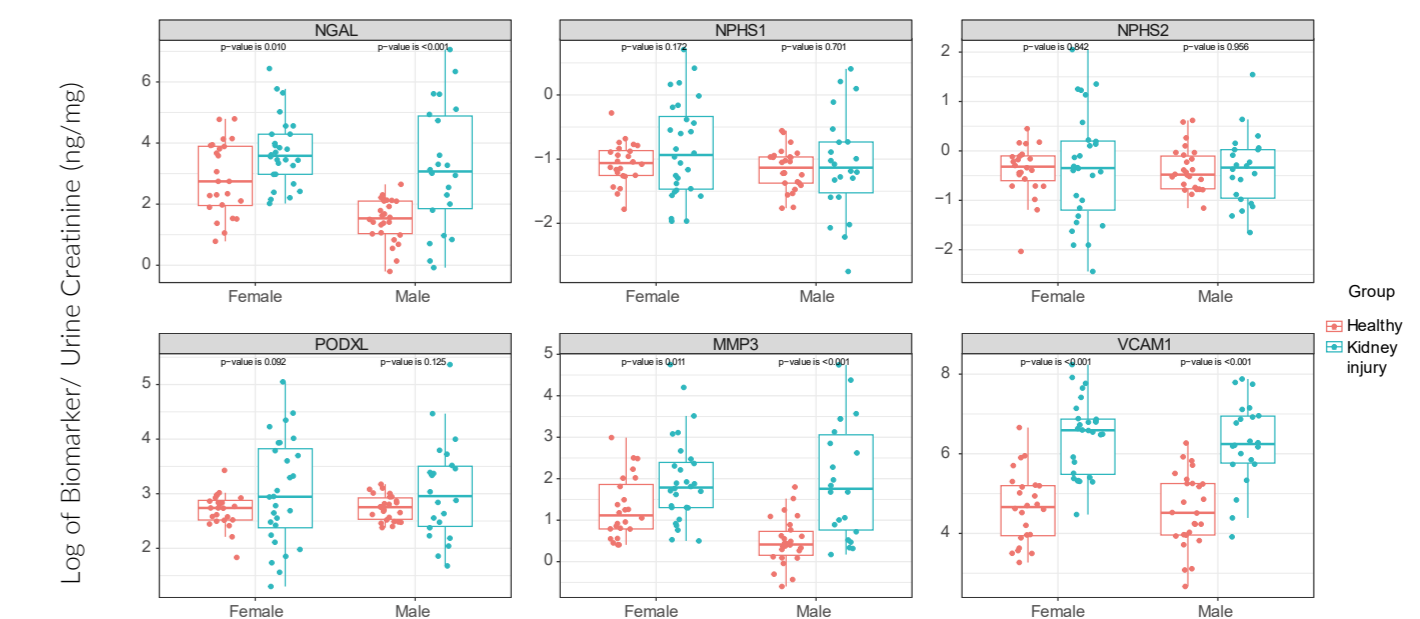


Figure 3: Analysis of the three glomerular proteins Nephritin (NPHS1), Podocin (NPHS2) and Podocalyxin (PODXL), the tubular injury protein biomarker Neutrophil gelatinase associated lipocalin (NGAL), and the two potential vascular markers Vascular cell adhesion molecule 1 (VCAM1) and Matrix metalloproteinase 3 (MMP3) in urinary samples of patients with glomerular damage (n=50, Kidney injury) and healthy volunteers (n=48).

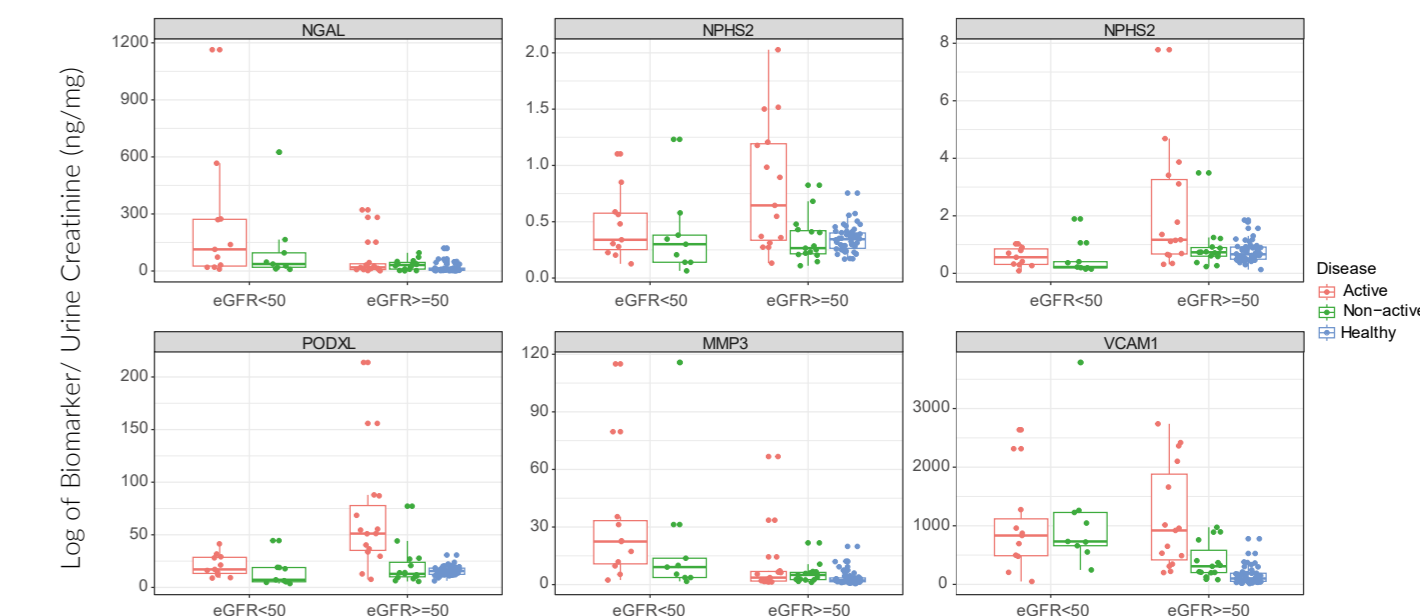


Figure 4: The diseased and healthy control subjects were divided into active and inactive disease subjects with eGFR ≥ 50 , reflecting normal filtration rate, and subjects with eGFR < 50 , reflecting abnormal filtration function.

SUMMARY & OUTLOOK

- Significant differences between patients with glomerular disease and control subjects for MMP3, NGAL, PODXL, and VCAM1, with VCAM1 showing the highest discriminatory performance.
- NPHS1, NPHS2, and PODXL were significantly increased in patients with mildly reduced renal function (eGFR ≥ 60) compared to controls.
- Longitudinal analysis of biospecimens to evaluate the temporal emergence of novel biomarkers in relation to the urine albumin-to-creatinine ratio.

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