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Assay validation and quantification of novel protein safety biomarker candidates for the detection of glomerular injury in clinical specimens

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ABSTRACT

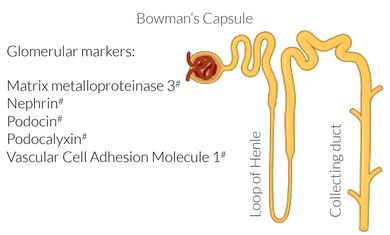
Biomarkers of kidney tubular injury have been extensively studied and qualified for nonclinical safety assessment as well as at a cohort level in healthy volunteers in phase 1 clinical trials if there is an a priori suspicion of nephrotoxicity. In contrast, data on the performance of urinary biomarkers of drug-induced glomerular injury are lacking. This is especially true for 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) project, which is supported by the EU Innovative Medicine Initiative program, aims to qualify biomarkers for drug-induced organ injury. One specific aim is the identification of novel glomerular injury biomarkers in patients at risk and demonstrating how a panel of glomerular specific biomarkers can provide pathomechanistic insights to drug-induced renal injury especially when used in conjunction with tubular injury biomarkers. This will facilitate detection of subclinical (= without alterations in serum-creatinine and progressive elevations of proteinuria) glomerular injury.

A multiplex assay based on immunoaffinity enrichment combined with mass spectrometry read out has been established for the glomerular protein biomarker candidates Nephrin (NPHS1), Podocin (NPHS2) and Podocalyxin (PODXL), seven tubular protein biomarkers and two vascular protein biomarkers (see detailed list in Figure 1).

Assays were applied in urinary samples from normal healthy volunteers (n=50) and from a patient cohort (n=50) with different underlying diseases (e.g. *Lupus Nephritis*) but histopathological confirmation of glomerular injury.

TUBULAR AND GLOMERULAR PROTEIN BIOMARKERS



Glomerular markers:

Matrix metalloproteinase 3#
Nephrin#
Podocin#
Podocalyxin#
Vascular Cell Adhesion Molecule 1#

Tubular markers:

α-1-Microglobulin
Clusterin*
Cystatin C*
Kidney Injury Molecule 1*
Neutrophil Gelatinase-Associated Lipocalin*
Osteopontin*
Retinol-Binding Protein 4

Accepted at FDA´s Biomarker Qualification Program
* Part of FDA qualified urinary nephrotoxicity biomarker panel

Figure 1: Nephrin (NPHS1), Podocin (NPHS2), Podocalyxin (PODXL) are glomerular injury biomarker candidates. α-1-Microglobulin (AMBP), Clusterin (CLU) Cystatin C (CST3), Kidney Injury Molecule 1 (KIM1), Neutrophil Gelatinase-Associated Lipocalin (NGAL), Osteopontin (OPN) and Retinol-Binding Protein 4 (RBP4) 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.

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METHOD - IMMUNOAFFINITY-LC-MS/MS

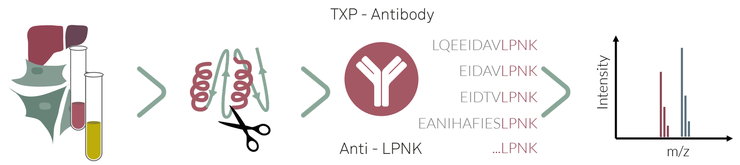

Figure 2: Workflow of immunoaffinity-LC-MS/MS. Urinary proteins are tryptically digested and isotopically labeled standard peptides are added. Group-specific antibodies targeting C-terminal sequence motifs are employed in an automated immunoprecipitation workflow to enrich peptides derived from proteins of interest. Finally unique peptides derived from the protein biomarkers are quantified via reference peptides measured by targeted nano LC-mass spectrometry.

We have established a protein assay platform for the analysis of renal injury biomarkers in clinical urine specimen. First, proteins are enzymatically fragmented with trypsin. Peptides derived from the biomarkers are enriched by the use of multi-specific antibodies (TXP antibodies) targeting common C-terminal amino acid motifs present in the sequences of the biomarkers, regardless of species. The immunoaffinity enriched peptides are detected and quantified by nano-liquid chromatography coupled to mass spectrometry (immunoaffinity-LC-MS/MS) with reference to synthetic isotope-encoded standards.

ASSAY VALIDATION

Validation was carried out according to FDA and EMA guidance documents. Reproducibility, parallelism, interference, accuracy and precision, and stability were evaluated using standards, biological quality control samples and patient samples. Exemplarily, part of the determination of inter assay accuracy and precision in biological controls and reproducibility results for the potential glomerular biomarker Podocalyxin (PODXL) are shown in Table 1 and 2. Assay accuracy and precision were assessed by the repeated analysis of biological QC samples (QC1, QC2, and QC3) in six independent analytical runs. The QC samples were generated by spiking urine from healthy donors with protein extracts from human kidneys.

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: ±20% ; at LLOQ/ULOQ ±25%, & Total Error ≤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 ±30%. Difference (%) was calculated as follows: 100 x (result $_{replicate\ 2}$-result $_{replicate\ 1}$) / result $_{average}$

	Donor (mean value, n=2), ng/mL														
Run	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 the urinary glomerular, tubular and vascular assays, we generated learning phase data from samples collected at a single time point from patients with histology-confirmed glomerular injury/disease (n=50) and normal healthy volunteers (n=50). Preliminary analysis suggests that median biomarker values are higher in glomerular injury/disease cases compared to controls for NGAL (5.7-fold), PODXL (2.1-fold), MMP3 (4.7-fold), and VCAM1 (5.9-fold). However, the distinction was not as clear for NPHS1 (1.5-fold), and NPHS2 (1.5-fold) between cases and controls (Figure 3). Moreover subgrouping of cases and controls by 50<eGFR≥50 and active and non-active disease improved separation in case of the glomerular protein biomarker candidates (Figure 4).

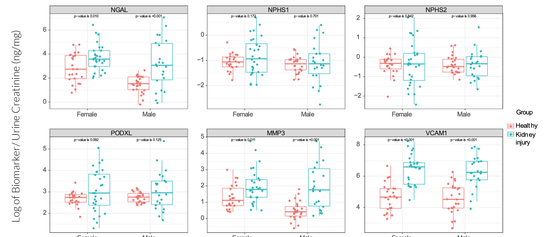

Figure 3: Analysis of the three glomerular proteins Nephrin (NPHS1), Podocin (NPHS2) and Podocalyxin (PODXL), the tubular injury protein biomarker Neutrophil gelatinase associated lipocalin (NGAL), and the two potential vascular markers Vascular cell adhesion molecule 1 (VCAM1) and Matrix metalloproteinase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

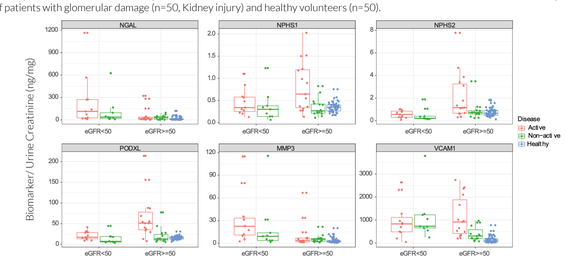

Figure 4: The diseased and healthy control subjects were divided into active and inactive disease subjects and subjects with eGFR ≥50, reflecting normal filtration rate, and subjects with eGFR<50, reflecting abnormal filtration function.

OUTLOOK

- Assessing the glomerular and the vascular protein biomarker candidates in urine samples collected from patients with preeclampsia as well as cancer patients treated with VEGF-inhibitors.
- Analyses of longitudinal biosamples to investigate temporal onset of novel biomarkers in relation to urine albumin-creatinine ratio.

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