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# *Development and validation of assay methods for novel mechanistic protein biomarkers of drug-induced liver injury (DILI)*

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## ABSTRACT

The lack of biomarkers to accurately detect drug-induced liver injury (DILI) and determine its prognosis continues to hinder early- and late-stage drug development and remains a challenge in clinical practice. The international Translational Safety Biomarker Pipeline (TransBioLine) project is currently investigating several mechanistic protein biomarkers for the characterization of DILI. Information on these biomarker candidates will complement established markers such as albumin, total bilirubin, transaminases and prothrombin time to improve the diagnosis and prediction of prognosis of DILI. Macrophage colony-stimulating factor 1 receptor (MCSF1R), osteopontin (OPN), high mobility group protein B1 (HMGB1), glutamate dehydrogenase (GLDH), keratin 18 (K18) and caspase-cleaved keratin 18 (ccK18) reflect apoptotic, necrotic and immunological processes that contribute to the pathogenesis of DILI (Fig. 1).

We present here the assay development and validation for the protein biomarker candidates HMGB1, MCSF1R, OPN and GLDH in a multiplex fashion using immunoprecipitation combined with a mass spectrometric redout in EDTA plasma (assay 1). Moreover, we present a fit-for-purpose assay validation for K18 (assay 2) and ccK18 (assay 3) using commercially available immunoassay kits in EDTA plasma.

Assays were applied in EDTA plasma samples from normal healthy volunteers (n=50) and from a patient cohort with different underlying diseases but suspected acute DILI.

## NOVEL MECHANISTIC PROTEIN BIOMARKERS OF DILI

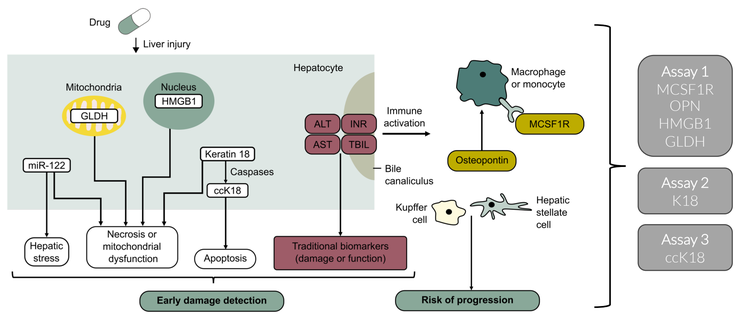


Figure 1: Assay 1 using 15 µL EDTA plasma per replicate capable of quantifying macrophage colony-stimulating factor 1 receptor (MCSF1R), osteopontin (OPN), high mobility group protein B1 (HMGB1), and glutamate dehydrogenase (GLDH). Assay 2 using 25 µL of EDTA plasma per replicate is capable of quantifying keratin 18 (K18). Assay 3 also using 25 µL of EDTA plasma per replicate is capable of quantifying caspase-cleaved keratin 18 (ccK18). This biomarker panel reflects apoptotic, necrotic and immunological processes that contribute to the pathogenesis of DILI.
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## ACKNOWLEDGMENT

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## METHOD - IMMUNOAFFINITY-LC-MS/MS

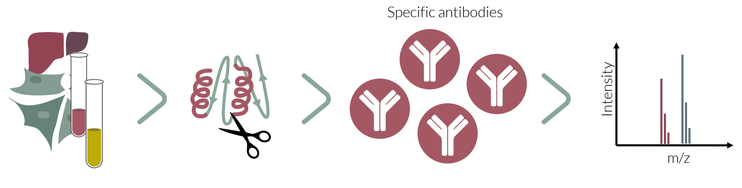


Figure 2: Workflow of immunoaffinity-MS. Plasma proteins are tryptically digested and isotopically labeled standard peptides are added. Sequence-specific antibodies targeting proteotypic sequence motifs are employed in an automated immunoprecipitation workflow to enrich peptides derived from proteins of interest and standards. Finally unique peptides derived from the protein biomarkers are quantified via reference peptides and targeted nLC-mass spectrometry.

For assay 1, we have established a protein assay platform for the analysis of novel mechanistic DILI biomarkers in clinical EDTA plasma samples. First, proteins are enzymatically fragmented with trypsin. Peptides derived from the biomarkers are enriched by the use of sequence-specific antibodies targeting proteotypic sequences within the biomarkers. 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. Accuracy, dynamic range (LLOQ, ULOQ), parallelism, precision, selectivity, reproducibility and sample stability were evaluated using standards, and biological quality control samples and patient samples. In addition, the effects of sample matrix and handling on the quantification of these novel protein biomarkers were investigated. Exemplarily, part of the determination of inter assay accuracy and precision in biological controls and reproducibility results for the novel mechanistic DILI biomarker OPN are shown in Table 1 and 2. Assay accuracy and precision were assessed by the repeated analysis of biological QC samples in six independent analytical runs. The QC samples were generated by spiking EDTA plasma from healthy donors with recombinant protein.

Table 1: Inter-assay precision and accuracy for OPN was determined by repeated analysis of biological quality control samples (n=12). Acceptance criteria were as follows: Accuracy 80%-120%; at LLOQ/ULOQ 75%-125%, Precision ±20% ; at LLOQ/ULOQ ±25%, & Total Error ≤40%

| Run | Unit | QC L | QC M | QC H |
|---|---|---|---|---|
| Mean value, n=12 | ng/mL | 32.11 | 418.88 | 2636.96 |
| SD | ng/mL | 3.8 | 54.6 | 165.3 |
| CV | % | 12 | 13 | 6 |
| Nominal value | ng/mL | 32.00 | 435.46 | 2531.93 |
| Accuracy | % | 100 | 96 | 104 |
| TE | % | 12 | 17 | 10 |

Table 2: Reproducibility of OPN analysis was tested in EDTA plasma samples from 7 healthy donors (n=2). In at least 80% of the samples the percentage difference for results from two analytical runs should be within ±30%.

| | | Donor (mean value, n=2) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Run | Unit | R1 | R2 | R3 | R4 | R5 | R6 | R7 |
| 1 | ng/mL | 96.85 | 81.40 | 76.83 | 85.56 | 116.32 | 85.86 | 67.45 |
| 2 | ng/mL | 109.33 | 97.66 | 93.09 | 87.57 | 108.20 | 104.10 | 78.22 |
| CV | % | 9 | 13 | 14 | 2 | 5 | 14 | 10 |

## RESULTS

Using the validated novel mechanistic DILI biomarker assays, we generated learning phase data from samples collected at a single time point from patients with suspected DILI (n=100), with fatty liver disease (NAFLD/AFLD, n=53), with psoriasis or rheumatoid arthritis (n=29) and healthy volunteers (n=50). Preliminary analysis suggests that median biomarker values are higher in suspected DILI cases compared to healthy controls for K18 (5.7-fold), ccK18 (10.5-fold), HMGB1 (6.9-fold), MCSF1R (12.5-fold), OPN (3.38-fold) and GLDH (11.4-fold). Similar values are reported for suspected DILI cases compared to non-DILI controls.

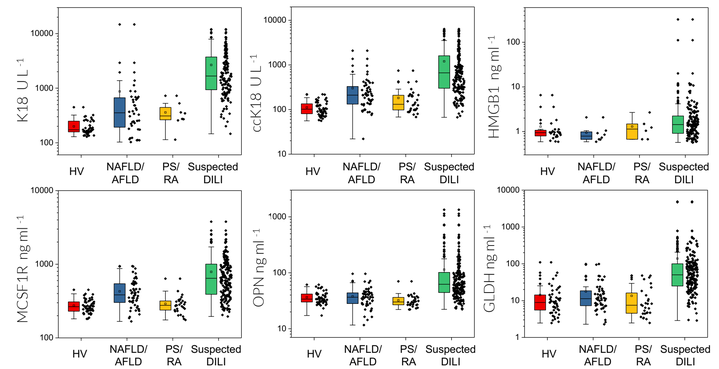


Figure 3: Analysis of the six novel mechanistic DILI biomarker proteins macrophage colony-stimulating factor 1 receptor (MCSF1R), osteopontin (OPN), high mobility group protein B1 (HMGB1), glutamate dehydrogenase (GLDH), caspase-cleaved keratin 18 (ccK18) and keratin 18 (K18) in EDTA plasma samples of patients with suspected DILI (n=50) with fatty liver disease (NAFLD/AFLD, n=53), with psoriasis or rheumatoid arthritis (n=29) and healthy volunteers (n=50). Biomarkers MCSF1R, OPN, GLDH and HMGB1 were assessed using a multiplexed immunoassay with mass spectrometric readout, biomarkers ccK18 and K18 were assessed using a commercially available immunoassay.

## SUMMARY AND OUTLOOK

As a next step, we are investigating whether the proteins or composite measures can be used as prognostic tools for DILI outcome. The prognostic value of selected protein biomarkers in idiosyncratic DILI will be statistically evaluated. Analysis of additional samples from DILI patients will continue after submission of a qualification plan and refined context of use to the FDA.

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