IMI TransBioLine
Drug-induced Liver Injury Work Package

Letter of Intent

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Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate transaminase
ccK18	Caspase-cleaved cytokeratin 18
DILI	drug-induced liver injury
DILIN	Drug-Induced Liver Injury Network
HMGB1	High mobility group box 1
IA-LC-MS/MS	Immunoaffinity-based liquid chromatography mass spectrometric assay
iDILIC	International DILI Consortium
IMI	Innovative Medicines Initiative
IMI LITMUS	Liver Investigation: Testing Marker Utility in Steatohepatitis
K18	Cytokeratin 18
MCSF1R	Macrophage colony-stimulating factor receptor 1
MELD	Model for End-Stage Liver Disease
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NIHR BRC	National Institute for Health Research Biomedical Research Centre
PSTC	Predictive Safety Testing Consortium
SAFE-T	Safer and Faster Evidence-based Translation
TASMC	Tel Aviv Sourasky Medical Center
TBIL	total bilirubin

1 ADMINISTRATIVE INFORMATION

1.1 Submission Title: Letter of Intent for the qualification of biomarkers of drug-induced liver injury

1.2 Requesting Information:

Requesting Organization

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1.3 Submission Dates:

LOI submission date: December 18, 2020

2 INTRODUCTION

Standard toxicological studies during the preclinical phase of drug development do not reliably detect the potential hepatotoxicity of a novel agent (Ballet F, 2015). Therefore, liver injury is one of the top three organ toxicities identified in phase I to III trials, leading to many promising drug candidates being dropped from clinical development. Hepatotoxicity has been the second most common reason for post-marketing withdrawals worldwide, accounting for 32% of 47 such drug withdrawals in three decades (Stevens JL, 2009). Lack of specific tests means that the diagnosis of Drug-Induced Liver Injury (DILI) is often delayed or left unrecognized (Aithal GP, 1999; M'Kada H, 2012). The annual incidence of DILI is 19 per 100,000, of which 23% are hospitalized and 1% die (Björnsson ES, 2013).

FDA and EMA issued Letters of Support to the IMI SAFE-T Consortium and to C-Path encouraging sponsors to implement new liver safety biomarkers in development programs for the assessment of DILI. The DILI Work Package (WP2) in the TransBioLine Consortium now plans to further develop biomarkers that can identify individuals with potential acute liver injury caused by drugs, in whom dose reduction or dose interruption is warranted. The study plan includes a learning and a confirmatory phase. For selected biomarkers, data from the learning phase are already available from the SAFE-T Consortium and these will be confirmed using the TransBioLine dataset.

3 DRUG DEVELOPMENT NEED STATEMENT

Idiosyncratic DILI affects 19 per 100,000 people and 1 in 5 are hospitalized due to symptoms. Once biliary obstruction is excluded, DILI is the second most common cause of jaundice; of those who are jaundiced, 10% die. Idiosyncratic DILI accounts for 7%-14% of acute liver failure cases in Europe (Bernal W, 2013) and rate of death and transplantation are consistently higher with idiosyncratic DILI compared to acetaminophen overdose. Biomarkers that could detect liver injury early in both clinical trials and routine practice would reduce serious consequences of DILI.

Biomarkers that distinguish adaptation, and therefore recovery, from progression and therefore serious liver injury, in DILI will transform monitoring in clinical trials and strengthen regulatory approval of novel molecular entities.

There is a strong need for biomarkers that could be used in drug development to identify and stratify patients who progress to develop acute liver failure or develop chronicity in the longer term. Concordance between standard toxicological studies performed today and idiosyncratic DILI in humans is poor. Tools to distinguish adaptation from potential DILI are lacking. The standard panel of liver laboratory tests performed today lack sensitivity and specificity and do not predict the clinical course of a patient in whom DILI is suspected. The TransBioLine Work

Package (WP) on biomarkers of liver injury is planning to qualify biomarkers that indicate a risk of progression from hepatocellular injury to severe Drug-Induced Liver Injury. The DILI WP will focus on acute idiosyncratic DILI and its outcomes as defined by Aithal GP, 2011.

4 Biomarker Information and Interpretation

4.1 Biomarker name

Standard biomarkers that will be measured to define DILI and assess liver function comprise ALT, AST, bilirubin, alkaline phosphatase, INR and albumin.

We plan to identify a subset of promising molecular biomarkers across the categories listed below, as well as miRNA biomarkers (see Table 4-1 for description of the biomarkers):

Table 4-1 Description of biomarkers of drug-induced liver injury

Marker	ID	Origin of Biomarker	Summary
High mobility group box 1 (HMGB1)	P09429	Detectable in almost all tissues	HMGB1 predicts patient prognosis following APAP overdose
Cytokeratin 18 full-length (K18)	P05783	Epithelial cells	The full-length protein is released from necrotic cells. It is significantly elevated in acetaminophen overdose patients that die/require a liver transplant compared to spontaneous survivors
Cytokeratin 18 Caspase-cleaved fragment (cc-K18)	P05783	Epithelial cells	The caspase-cleaved fragment is released from apoptotic cells and helps define the type of cytotoxicity. cc Keratin 18 fragments in blood predict severity of disease in NASH and in hepatitis C.
MicroRNAs		miR-122 is liver-specific	MicroRNA 122 is an early marker of hepatocellular injury, possibly preceding ALT on a temporal scale, and is a specific marker of hepatocellular injury. It has been reported as a sensitive DILI marker in multiple clinical studies

Marker	ID	Origin of Biomarker	Summary
Glutamate dehydrogenase (GLDH)	P00367	Mitochondrial matrix; primarily in the centrilobular region of the liver; lower levels in the kidney and brain	A sensitive biomarker of liver toxicity with hepatocellular damage in preclinical species; shown to be elevated in humans with hepatic ischemia or hepatitis; shown to correlate with ALT in patients with a broad range of clinically demonstrated liver injuries including acetaminophen-induced liver injury and to detect mild hepatocyte necrosis in patients treated with heparin. Marker for mitochondrial injury or cellular injury in multiple clinical DILI and acute liver failure studies
Osteopontin (OPN)	P10451	Multiple tissue and cell types including liver	Elevated serum levels of OPN are detectable in patients with severe liver damage. Increased levels of serum OPN are associated with a poor prognosis. Plasma OPN levels in fulminant hepatic failure patients were higher than those of acute hepatitis patients and healthy adults. OPN is associated with inflammatory cell activation and with liver regeneration due to activation of hepatic stem cells
Macrophage colony Stimulating factor receptor 1 (MCSF1R)	P07333	Cytokine receptor on macrophages/monocytes	Data from the ximelagatran biomarker discovery study suggest that MCSF1R is shed from macrophages during DILI. CSFR1 serum/plasma levels may have value as a prognostic marker for liver disease associated with inflammation.
Bile acids	----	Synthesized by the liver	1) early markers of cholestasis, possibly preceding ALP and ALT on a temporal scale 2) sensitive marker of inhibition of the bile salt export pump (BSEP), known to be inhibited by several drugs 3) marker of liver synthetic function
Sphingolipids	----	Abundant in the liver	Increased sphingolipid levels in plasma coincide with liver dysfunction

The following **protein biomarkers** listed in Table 4-1 will be used in the Context of Use proposed in section 5:

- High mobility group box 1 (HMGB1)
- Cytokeratin 18 (CK18); (molecular biomarker)
- Caspase-cleaved cytokeratin 18(ccCK18)
- Macrophage colony-stimulating factor receptor 1(MCSF1R)
- Osteopontin (OPN)
- Glutamate dehydrogenase (GLDH)

The following exploratory biomarkers listed in Table 4-1 will also be assayed, although the results are currently not part of the dataset supporting the Context of Use:

Bile acid profile

Bile acids were already studied in the context of the IMI SAFE-T Consortium and selected bile acids – as identified in the LC-MS spectra of patients with acute DILI – showed promise in identifying severe idiosyncratic DILI. These data were contained in the final submission package of the SAFE-T Consortium submitted to FDA and EMA on January 4, 2016, but were not published. In the TransBioLine Consortium, bile acids will be profiled using the same technology, but in a different laboratory. A recent publication (Ma Z, 2019) found significant changes in the levels of selected bile acids in patients with DILI vs healthy controls ($P<.001$). The authors also showed that the severity of liver damage correlated with the observed changes in the serum concentrations of selected bile acids.

Sphingolipids

Sphingolipids are abundant in hepatocytes and 1-deoxysphingolipids have been shown to be increased in patients with non-alcoholic fatty liver disease (Gai Z, 2020). Sphingolipid profiles are also altered in alcoholic liver disease, hepatocellular carcinoma and intrahepatic cholestasis. However, little data exists on sphingolipid profiles in DILI. Sphingolipid profiles will initially be measured in the first 100 DILI patients recruited; the total recruitment aim is 260 DILI cases. The first 100 patients will be considered an exploratory dataset. Depending on the results of the interim analysis, the remaining 160 DILI cases may be used as a confirmatory dataset to specifically assess defined sphingolipid species.

miRNAs: circulating microRNAs

MicroRNA profiling will be performed as part of the overall liquid biopsy strategy that is being implemented in all five organ work packages in TransBioLine. MiRNA-122 is a known hepatocyte-specific miRNA that was already assessed in the SAFE-T Consortium and was recommended for further investigation as a biomarker of liver specific injury in patients with acute DILl in the FDA Letter of Support to the SAFE-T Consortium dated July 25, 2016.

4.2 Analytical methods

Protein biomarkers will be measured on two technical platforms (a) by immunoaffinity-based liquid chromatography mass spectrometric read-out (IA-LC-MS/MS) and (b) standard sandwich Immunoassays (see Table 4-2).

The proteins **HMGB1**, **OPN**, **MCSF1R** and **GLDH** will be analyzed in multiplex fashion by IA-LC-MS/MS. Here, plasma proteins are enzymatically fragmented into peptides first. Then, antibodies are employed to enrich peptides derived from the proteins of interest which can be used to unambiguously identify the protein (proteotypic). Each protein will be quantified by adding $^{13}C/^{15}N$-labelled peptide standards to the fragmented sample before the immunoprecipitation. The peptides are measured as protein surrogates by parallel reaction monitoring mass spectrometry. The quantification is achieved by stable isotope dilution (internal $^{13}C/^{15}N$-labelled peptide standard). The readout for the potential protein biomarkers listed in Table 4-2 is performed using an Orbitrap mass analyzer (QExactive plus, ThermoFisher). Peptide quantification will be based on the calibration curve obtained by peak area ratio (analyte/ internal standard) using a logistic regression model. Protein concentrations are calculated by converting peptide amount (fmol) into ng ml^{-1} considering the molecular weight of the corresponding protein.

The proteins **CK18** and **ccCK18** will be analyzed by running standard sandwich immunoassays. Here a commercially available kit will be employed. In brief, proteins will be captured by a capture antibody and detected by an enzyme-antibody conjugate.

Bile acids will be analyzed using HPLC-MS/MS.

Sphingolipids are analyzed by liquid chromatography-mass spectrometry (LC-MS).

4.3 Measurement units and limit(s) of detection

Reference intervals for selected biomarkers were published based on measurements obtained in healthy volunteers (HVs) in two different cohorts (Church RJ and Kullak-Ublick GA, 2019). Samples from eighty-one (81) HVs were obtained through the Predictive Safety Testing Consortium (PSTC) and 192 HVs were obtained through the Tel Aviv Sourasky Medical Center (TASMC). The Tel Aviv samples were obtained through the IMI SAFE-T Consortium (Safer and Faster Evidence-based Translation), in which TASMC was a partner. The biomarker reference intervals between PSTC and SAFE-T showed substantial overlap (Church R, 2019).

Reference intervals were obtained for the following biomarkers that are being evaluated in the DILI WP of TransBioLine:

- K18 (U/L)
- ccK18 (U/L)
- MCSF1R (ng/mL)
- Osteopontin (ng/mL)
- GLDH (U/L)
- Micro-RNA 122 (copies/µl)

For HMGB1, bile acids and sphingolipids, reference ranges will be obtained in 100 HVs that will be provided by an industry partner. Two aliquots from each HV are available.

Measurement units and LLOQ for the Biomarkers are given in Table 4-2 below.

Table 4-2 Assay platforms, dynamic range and LLOQ of DILI-biomarker assays

Biomarker	Analysis platform	Matrix	Dynamic Range (ng/mL) (*U/L)	LLOQ (ng/mL) (*U/L)	Standard Inter-Assay Precision, %CV Range (n=6)
Total HMGB1	IA-LC-MS/MS	EDTA-Plasma	0.57 – 138	0.57	2.4-11.7
MCSF1R	IA-LC-MS/MS	EDTA-Plasma	17.6 – 5698	17.6	2.8-8.2
OPN	IA-LC-MS/MS	EDTA-Plasma	2.7 – 3540	2.7	3.5-13.9
GLDH	IA-LC-MS/MS	EDTA-Plasma	2.6 – 1866	2.16	4.7-17.6
K18	Sandwich Immunoassay	EDTA-Plasma	100 – 5000*	100*	2.2-10.4
ccK18	Sandwich Immunoassay	EDTA-Plasma	38 – 100*	38*	3.0-17.9

4.4 Biomarker interpretation and utility

Based on the previous work performed in the SAFE-T Consortium and published in Hepatology in (Church R, 2019) a full exploratory dataset is available for six of the biomarkers under investigation in TransBioLine. Reference ranges previously determined in two independent cohorts of HVs will be validated in 100 HVs. Blood levels of these biomarkers will be assessed in 260 patients with DILI and compared with various control groups of NAFLD, AFLD, non-DILI controls and methotrexate-treated patients. The biomarkers may allow prospective monitoring of patients in whom an initial DILI diagnosis has been established. Univariate and multivariate combination approaches will be used to assess biomarker levels in patients who fulfill the criteria for DILI. By being able to identify patients with DILI in whom biomarker levels exceed the DILI threshold, the biomarkers would thereby facilitate decisions to interrupt or reduce dosing Biomarkers could also allow the continuation of dosing in situations where the standard marker ALT is elevated due to causes not related to liver injury.

The threshold above which plasma biomarkers indicate DILI that warrants dose modification will be derived from biomarker levels measured in patients who fulfilled the following criteria:

- Acute liver injury caused by a drug, with causality adjudicated as “probable“
- ALT or AST ≥ 8x ULN

5 Context of Use Statement

Identification of patients with DILI in whom dose modification is warranted.

A single biomarker or a composite panel of biomarkers will aid in identifying patients with potential acute liver injury caused by drugs, in whom dose reduction or dose interruption is warranted. Acute liver injury is suspected based on elevations of alanine transaminase (ALT), aspartate transaminase (AST) or alkaline phosphatase (ALP).

6 Analytical Considerations

6.1 Biomarker measurement

Plasma levels of HMGB1, OPN, MCSF1R and GLDH will be analyzed in multiplex fashion by IA-LC-MS/MS, while the proteins CK18 and ccCK18 will be analyzed by running standard sandwich immunoassays as described in Section 4.2 Analytical Methods. Standard sandwich immunoassay kit is obtained from PEVIVA (M30 and M65 ELISA kit).

6.2 Index/scoring system

Based on the exploratory data generated within the SAFE-T Consortium, levels of OPN, CK18, MCSF1R and ccK18 significantly predicted progression of DILI to severe DILI as defined by death or liver transplantation. INR was found to have the strongest association with death/transplantation (AUC = 0.920) followed by OPN (AUC = 0.858). The biomarkers had more than 2-fold and more than 7-fold changes over DILI patients who did not experience liver failure and healthy volunteers, respectively.

The values of K18 and ccK18 measured in 98 DILI patients from the US DILIN network within SAFE-T enabled the calculation of a so-called apoptotic index (AI), defined as the ratio of ccK18:K18. The AI was significantly reduced in patients who died or required liver transplantation.

Another prognostic model was developed by integrating the biomarkers MCSFR1 and K18 into the so-called MELD score (Model for End-Stage Liver Disease), for the prediction of outcome in patients with acute DILI (Church R, 2019). A Model MELD score of ≥ 20 was highly sensitive and a MELD score ≥ 30 was highly specific for a prognosis of death/transplantation in acute idiosyncratic DILI. Incorporating K18 and MCSFR levels into the MELD score (when MELD values were in the intermediate range between 20-29) improved the specificity of using MELD score ≥ 20 (specificity of 0.889 when incorporating K18 and MCSFR with MELD score ≥ 20 versus 0.738 with MELD score ≥ 20 alone) without reducing the sensitivity of using MELD score ≥ 20 alone (sensitivity of 0.933 for both). These are examples of how the biomarkers that were analyzed in SAFE-T could be combined to yield an indexing/scoring system. In TransBioLine, the use of these biomarkers in identifying patients with DILI in whom dose modification or interruption is warranted will be assessed.

6.3 Sample source/matrix

Potential Biomarkers will be quantified from EDTA-Plasma. No additives or stabilizers need to be added. Stability of biomarkers will be analyzed during validation plan.

6.4 Pre-analytical stability and quality assurance/quality control

Pre-analytical stability in EDTA-plasma and serum has been tested. The six proteins are stable in EDTA-plasma without additives. Three biological quality controls will be used for in-study validation.

6.5 Analytical validation reports

The following validation reports for the immunoassay kits for K18 and ccK18 (Section 11.2.1: *Validation report K18 ccK18*), as well as for that of the IA-LC-MS/MS for MCSF1R, HMGB1, OPN, and GLDH (Section 11.2.2: *Validation report OPN HMGB1 MCSF1R GLDH*) are provided with this document:

- Study title: Validation of Keratin 18 and caspase-cleaved Keratin 18 immunoassay kits for the determination of Keratin 18 and caspase-cleaved Keratin 18 concentration in human plasma
- Study title: Validation of an immuno-LC-MSMS based assay for the quantification of Macrophage Colony Stimulating Factor 1 Receptor (MCSF1R), High mobility group protein B1 (HMGB1), Osteopontin (OPN), and Glutamate Dehydrogenase 1 (GLDH) from human plasma.

6.6 Analytical validation plan for final version of the measurement tool

The Validation Plans have been developed by TransBioLine members. The Validation was executed by Signatope in the laboratory of Signatope. Validation results were approved by an independent scientific monitor. See Validation Reports (Section 11.2.1 and Section 11.2.2).

7 Clinical Considerations

7.1 Use in Drug Development

Identifying biomarkers of DILI would represent a major advancement in patient safety towards the prevention and better management of this safety risk. Early measurement of the appropriate biomarker(s) could identify idiosyncratic DILI with a risk of progression to severe DILI, and thereby alter the subsequent clinical management and risk stratification of such cases. This would result in more efficient, safer drug development with reduced frequency of abandoning the development of promising drugs.

A decision tree showing at which stage and in what context biomarkers could be implemented in clinical trials to facilitate decision-making with regards to dose modification or interruption is shown in Figure 7-1.

7.2 Patient population or drug development setting

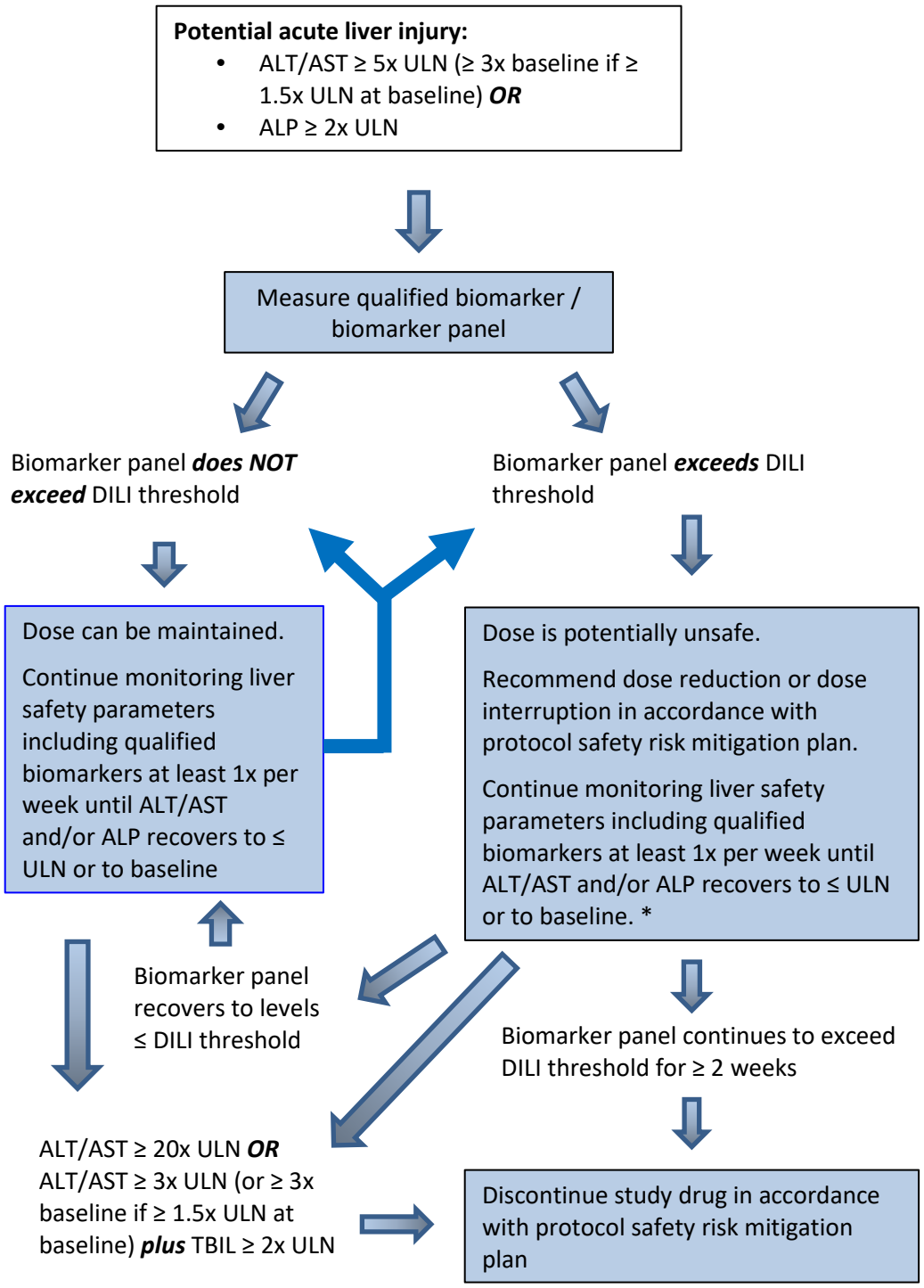
The biomarkers are being evaluated for individuals participating in clinical trials in whom liver injury caused by an investigational drug is suspected. Idiosyncratic liver injury is unpredictable and can occur in any phase of clinical development. The laboratory triggers include an elevation of ALT or AST to ≥ 5x ULN, which is the threshold for defining DILI. If bilirubin levels rise to ≥ 2x ULN simultaneously or within a reasonable time frame after an initial elevation of ALT/AST to ≥3x ULN, the case is considered a case of special interest that warrants further investigation. If causality of the suspected drug is assumed with a probability of ≥ 50%, the case is considered to fulfill Hy's law criteria, implying a risk for progression to liver failure of 10%. This usually leads to permanent discontinuation of the investigated drug, in line with the FDA Guidance for Industry.

The FDA Guidance also recommends discontinuation of treatment when the following thresholds are reached:

- ALT or AST >8x ULN
- ALT or AST >5x ULN for more than 2 weeks

These criteria have been adopted by pharmaceutical industry as "stopping rules" in drug development, provided that adjudication of the individual case indicates causality of the suspected drug. The difficulty in basing stopping rules on ALT/AST alone is that the magnitude of elevation of ALT/AST has little prognostic value in predicting the further clinical course of liver injury, for instance progression to severe DILI. It is, therefore, unclear whether stopping a potentially beneficial drug based on an 8x ULN ALT is in the patient's best interest from a safety perspective. The new biomarkers could complement ALT and/or AST in confirming that the degree of liver injury is sufficient to warrant treatment discontinuation / dose reduction.

Figure 7-1 Decision tree for use of the DILI biomarker panel in clinical development, as part of the clinical safety plan in study protocols



* If dose was interrupted, readminister at reduced dose level after resolution.

7.3 Clinical validation

In March 2018, the European Medicines Agency withdrew the drug flupirtine after 34 years of marketing and more than 12 million patients exposed, due to several hundred documented cases of DILI and more than 50 cases of liver failure. One of the biomarkers under study in TransBioLine – MCSF1R – was found to be highly elevated in 14 cases of flupirtine-induced DILI, and blood levels of this biomarker were in fact one log order higher in flupirtine-induced DILI than in acetaminophen-induced liver injury (Church R, 2019).

In TransBioLine, we will build on the discoveries made by global research programs such as the International DILI consortium (iDILIC), key biomarkers identified by IMI SAFE-T and the network established by the Pro-Euro DILI Registry; in addition, we have access to large datasets and samples from Drug-Induced Liver Injury Network (DILIN), Innovative Medicines Initiative Liver Investigation: Testing Marker Utility in Steatohepatitis (IMI LITMUS) and the National Institute for Health Research Biomedical Research Centre (NIHR BRC). The results of the IMI SAFE-T DILI Work Package clearly showed that selected biomarkers offer potential as diagnostic tools for the management of DILI. For example, MCSF1R and several bile acids showed high levels in flupirtine-induced DILI, a prototype idiosyncratic form of DILI (Church R, 2019). In TransBioLine, we will build on these findings by measuring these biomarkers in patients with acute DILI as well as control patients, the clinical protocols are listed below. The thresholds identified in the exploratory SAFE-T dataset will be validated in the TransBioLine DILI protocols. We will also assess performance of biomarkers in other forms of liver injury such as alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH). Drug-induced liver injury that leads to ongoing chronic liver damage with onset of fibrosis is an additional category that will be investigated in patients receiving methotrexate. The strategy of combining high quality clinical data from patients with severe DILI with biological material collected prospectively through the course of liver injury, and powerful bioinformatics/analytical tools will provide a non-biased platform for biomarker discovery, validation and regulatory qualification. The multidisciplinary collaborative approach of TransBioLine provides an ideal environment for innovation.

7.3.1 Aims/Goals

The TransBioLine DILI Work Package plans to:

- Validate the reference ranges in healthy volunteers established previously in the SAFE-T dataset, determine intra- and inter-subject variability
- Validate and qualify the safety biomarker panel using the following patient cohort:
  - 300 cases of DILI
  - 130 non-DILI acute controls
  - 100 cases of Alcoholic liver disease (ALD)

- 100 cases of Nonalcoholic fatty live disease (NAFLD)
- 100 healthy controls

7.3.2 Studies

The following studies will be conducted:

1. **TransBioLine Pro-Euro-DILI Registry: Creation of a multicentre and multidisciplinary European registry of prospective drug-induced liver injury cases** (Section 11.2.3)

   Specific objectives:

   - To set-up an international European registry of patients with idiosyncratic DILI, (iDILI) enrolled prospectively with the collation of in-depth phenotyping: including details of drug dose, duration, concomitant medications, host demography, comorbidity including insulin sensitivity as well as the course of the event
   - To enroll a similarly well-characterised control group of people who are exposed to medications matched for those implicated in DILI
   - To additionally form a cohort of patients with DILI-like symptoms who appear to be have DILI on presentation but who are subsequently found to have other causes for the symptoms
   - To collect and store biological samples (blood, urine, stool and liver biopsy) from patients with suspected idiosyncratic DILI and the symptomatic non-DILI control group through the course (at onset and follow-up) of the event
   - To collect and store biological samples (blood, urine and stool) from control patients at a single visit
   - To determine and analyze individual phenotypes and biochemical and cellular component levels in patients and control groups in order to develop translatable disease biomarkers via collation of inter-disciplinary data in the TransBioLine database

2. **Case-control Study Evaluating Biomarkers and Genetic Factors Associated with the Development of Non-alcoholic Steatohepatitis (NASH) and Alcoholic Steatohepatitis (ASH)** (Section 11.2.4)

   Research Aims:

   - To evaluate the role of non-invasive biomarkers in the assessment of non-alcoholic steatohepatitis and alcoholic steatohepatitis
   - To determine and analyze individual phenotypes and biochemical and cellular component levels in patients and control groups in order to develop translatable disease biomarkers via collation of inter-disciplinary data in TransBioLine Database

- NAFLD and ALD patients are important causes of raised liver enzymes in clinical trials as well as clinical practice. Biomarkers that are used to identify DILI have to be tested against these common chronic conditions

3. **Evaluation of the role of AST/ALT ratio, ELF markers and Fibroscan in the detection of methotrexate-induced hepatotoxicity** (Section 11.2.5)

   This protocol describes the evaluation of the role of AST/ALT, novel liver injury markers and Fibroscan in the detection of methotrexate induced hepatotoxicity. The study will investigate the role of novel biomarkers in the detection of methotrexate associated liver disease in patients with psoriasis and rheumatoid arthritis. The data will inform an optimal pathway for monitoring of liver disease in these patients, thereby reducing the number of liver biopsies routinely performed.

These studies will allow a comparison of biomarker levels measured in patients with acute idiosyncratic DILI with those measured in patients with non-drug induced liver injury, nonalcoholic steatosis/steatohepatitis as well as alcoholic steatohepatitis. Moreover, the performance of the DILI biomarkers in chronic forms of liver injury such as methotrexate-induced hepatotoxicity will be assessed. The results should allow conclusions to be drawn as to whether the new biomarkers predict progression to severe liver injury caused by the drug in question and whether the levels of the biomarkers correlate with the severity of the subsequent liver injury. For this reason severe forms of non-drug induced liver injury such as alcoholic steatohepatitis represent a useful control group.

7.3.3 Statistical analysis plan

Descriptive statistics, median and interquartile range, will be used to describe continuous variables, and frequency and percent will be used to describe categorical variables. All statistical analyses will be performed using R (R Core Team) or SAS software (Cary, NC). Biomarker distributions will be visualized to establish whether they display a log normal distribution. For consistency, the absolute value of all biomarkers will be log-transformed for statistical analyses. Statistical significance is considered if $P < 0.05$.

7.3.3.1 Reference ranges for healthy controls

Reference ranges for each of the biomarkers will be established in the 100 healthy controls. The goal is to assess variability of each biomarker measured within (using longitudinal measures for all the visits) and across subjects to define properties of variability and reproducibility in healthy subjects. Differences due to sex, ethnicity, age group, and recruitment center will be evaluated. If there is evidence of differences among the groups, then separate reference ranges will be constructed.

The inter-and intra-subject coefficients of variations will be calculated to quantify variability over time periods and between subjects.

The normality will be checked using Shapiro-Wilk's tests, res*pective*ly. If normality holds, then parametric 2.5th and 97.5th percentiles will be used for reference ranges; if normality does not hold, non-parametric 2.5th and 97.5th percentiles will be used. Prior to the reference ranges, outliers will be identified using both statistical method (e.g., from the box plot) and biological adjudication.

The reference ranges will be compared to the dynamic ranges in Table 4-2 using appropriate parametric or non-parametric tests depending on the biomarker distributions.

7.3.3.2 Biomarkers of DILI diagnosis

Biomarker differences in different patient cohorts will be determined using a one-way ANOVA with Tukey multiple comparison correction. Correlation of each biomarker with ALT will be determined using Pearson's r coefficients.

Receiver operator characteristic (ROC) curve analysis will be utilized to determine each of the six candidate biomarkers in Table 4-2 as well as their combinations for detection of DILI patients. Biomarkers are considered predictive of DILI if both the ROC area under the curve (AUC) and the lower end of the 95% confidence interval (CI) are >0.5. For this analysis the 100 healthy controls will always be used as controls, and each of the first four patient cohorts in section 7.3.1 will be used as cases for each ROC analysis. The primary case cohort of interest is the DILI cohort. Multi-variate ROC (i.e., using a composite measure of biomarkers) will be based on the multiple logistic regression.

7.4 Benefits and risks

Identifying safety biomarkers of DILI would represent a major advancement in patient safety towards the prevention and better management of this safety risk. Early measurement of these biomarkers could identify idiosyncratic DILI and thereby alter the subsequent clinical management and risk stratification of such cases. Conversely, the risk of over-interpreting biomarker findings could lead to false clinical decision-making and premature termination of an otherwise promising study drug. Because standard liver safety monitoring continues to be implemented throughout any biomarker study, assessing new biomarkers is unlikely to confer any risk to patients before firm conclusions on the clinical use and interpretation of new biomarkers have been reached. The aim in the current proposal is to position the biomarkers under investigation in the spectrum of phenotypic manifestations of drug-induced liver injury.

To mitigate risk, we will continue to implement Hy's law as the "gold standard" of predicting a risk of progression to liver failure as well as an ALT ≥ 20x ULN as a cutoff for immediate termination of study treatment. Cases consistent with Hy's law are marked by peak ALP levels <2x ULN and/or nR values ≥ 5. The "new R" (nR) value is defined as the ratio of either ALT or

AST (whichever is higher) to ALP and is expressed as multiples of their ULN (Robles-Diaz M, 2014).

7.5 Current knowledge gaps, limitations, and assumptions

The biomarkers under evaluation have received regulatory support for further use as exploratory biomarkers for the assessment of drug-induced liver injury. The identification of a biomarker that has true prognostic value in predicting the clinical course of a patient with as complex a phenotype as drug-induced liver injury is a high bar that would clearly represent a major breakthrough in the management of this deleterious adverse drug reaction. Whereas several potential mechanisms for DILI have been postulated, idiosyncratic DILI involves an immune component which is difficult to diagnose or anticipate based on currently available laboratory parameters. The biomarkers under investigation are therefore being assessed as safety rather than as prognostic biomarkers. In a patient with an ALT/AST $\geq$ 5x ULN, the biomarkers can serve to confirm the onset of drug-induced liver injury. A potential risk of the current approach is that not all potential mechanisms of DILI are reflected in the biomarkers under study, such that certain forms of DILI may not be picked up by the current biomarkers (for example oxidative stress in hepatocytes).

8 Supporting Information

The data from the SAFE-T Consortium were submitted on January 4, 2016; the FDA issued a Letter of Support on July 25, 2016 [https://www.fda.gov/media/99856/download]. The data were also published in Church RJ and Kullak-Ublick GA, 2019.

9 Previous Qualification Interactions and Other Approvals (if applicable)

9.1 Interactions with Food and Drug Administration

Previous Health Authority interactions regarding DILI biomarkers were held by the IMI SAFE-T consortium. In 2016 FDA issued a Letter of Support to the SAFE-T consortium encouraging evaluation of the following biomarkers for assessment of the risk of progression of DILI:

Cytokeratin 18 (CK-18), Total and hyperacetylated high mobility group protein B1 (HMGBI), Osteopontin, Macrophage colony-stimulating factor I receptor (CSF I R). The Agency also noted that the biomarkers micro RNA-122 (miR-122) and glutamate dehydrogenase (GLDH) deserved careful attention and could be studied further. TransBioLine is building on the work done by the SAFE-T consortium in evaluating the use of the biomarkers listed in Section 4.1 as biomarkers for drug-induced liver injury as proposed in this Letter of Intent.

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9.2 Interactions with European Medicines Agency

9.2.1 Letter of Support

The European Medicines Agency issued a Letter of Support for drug-induced liver injury (DILI) biomarkers on 30 September 2016 for the following proposed context of use statements:

Context-of–use statement "A":

Based on preliminary data, the following biomarkers have the potential as clinical DILI biomarkers that sponsors may choose to incorporate into their clinical trials to assess whether biomarkers provide additional information beyond the diagnostic value of ALT & TBIL according to the following mechanisms in the pathophysiology/pathogenesis (including the detection of severe DILI as defined by Hy's law criteria):

a. hepatocyte necrosis (CK-18, miR-122, total HMGB1, GLDH, SDH)
b. apoptosis (ccCK-18)
c. immune activation (hyperacetylated HMGB1, MCSF1R)

Context-of-use statement "B":

Based on preliminary data, the biomarkers hyperacetylated HMGB1, Osteopontin, Total Keratin 18 and MCSF1R have potential as clinical DILI biomarkers that sponsors may choose to incorporate into their clinical trials to anticipate early risk for progression of hepatocellular injury to severe DILI in patients in whom an initial DILI diagnosis has been established based on elevations of the standard marker ALT alone or in combination with TBIL.

Context-of-use statement "C":

Based on preliminary data, the following biomarkers: total HMGB1, total and caspase-cleaved keratin 18, miR-122, and GLDH have potential as clinical safety biomarkers that sponsors may choose to incorporate early (within the first 24 hours) in early stage clinical trials for the assessment of suspected intrinsic liver injury before ALT increases.

9.2.2 Interactions regarding acetylated HMGB1

Based on the recent revelation of potential scientific misconduct performed by an academic investigator at the University of Liverpool, who was responsible for the measurement of the biomarker acetylated HMGB1 (one of the biomarkers listed in the Letter of Support issued by EMA and FDA), EMA retracted its Letter of Support. The development of an analytical assay to

measure this biomarker is underway in several laboratories, but the biomarkers will not be included in the biomarker panel under study in TransBioLine until a robust analytical assay is available and pilot data support its potential use as a biomarker in DILI.

10 References

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11 Attachments

11.1 List of publications

Church, R.J., et al., Candidate biomarkers for the diagnosis and prognosis of drug-induced liver injury: An international collaborative effort. Hepatology, 2019. **69**(2): p. 760-773 [https://aasldpubs.onlinelibrary.wiley.com/doi/epdf/10.1002/hep.29802]

11.2 Other supporting information

11.2.1 Validation of Keratin 18 and caspase-cleaved Keratin 18 immunoassay kits for the determination of Keratin 18 and caspase-cleaved Keratin 18 concentration in human plasma

11.2.2 Validation of an immuno-LC-MSMS based assay for the quantification of Macrophage Colony Stimulating Factor 1 Receptor (MCSF1R), High mobility group protein B1 (HMGB1), Osteopontin (OPN), and Glutamate Dehydrogenase 1 (GLDH) from human plasma.

11.2.3 TransBioLine Pro-Euro-DILI Registry: Creation of a multicentre and multidisciplinary European registry of prospective drug-induced liver injury cases

11.2.4 Case-control Study Evaluating Biomarkers and Genetic Factors Associated with the Development of Non-alcoholic Steatohepatitis (NASH) and Alcoholic steatohepatitis (ASH)

11.2.5 Evaluation of the role of AST/ALT ratio, ELF markers and Fibroscan in the detection of methotrexate-induced hepatotoxicity