TransBioLine
Translational Safety
Biomarker Pipeline

Innovative Medicines Initiative - TransBioLine
Drug-induced Pancreas Injury Work Package

Letter of Intent

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CONFIDENTIAL

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Abbreviations

AP	Acute pancreatitis
CAPAP	Carboxypeptidase B activation peptide
CPB1	HUGO gene name for Carboxypeptidase B activation peptide
COU	Context of Use
CRP	C-reactive protein
CPA	Carboxypeptidase A
DIPI	Drug-Induced Pancreas Injury
ELISA	Enzyme-linked immunosorbent assay
IL-6	Interleukin 6
IMI	Innovative Medicines Initiative
IP	Immunoprecipitation
IP-LC-MS/MS	Immunoprecipitation coupled to nano-liquid chromatography tandem mass spectrometry
KUM	Klinikum der Universität München, Germany
LBA	Ligand binding assay
LC	Liquid chromatography
LLOQ	Lower limit of quantification
LNA	Locked nucleic acid
LOI	Letter of Intent
MAP-I	Markers in Acute Pancreatitis-I
MiRNA	Micro-ribonucleic acid
NGS	Next generation sequencing
NHV	Normal healthy volunteer
NPV	Negative predictive value
MRM	Multi reaction monitoring
PPV	Positive predictive value
PRSS1	Serine Protease 1 (HUGO gene name for Trypsinogen activation peptide)
PSTC	Predictive Safety Testing Consortium
RAC	Revised Atlanta Criteria
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SAFE-T	Safer and Faster Evidence-based Translation
SAP	Statistical analysis plan
STARD	Standards for reporting diagnostic accuracy
TAP	Trypsinogen activation peptide
ULIV	University of Liverpool, UK
ULOQ	Upper limit of quantification
UMA	Universidad de Málaga, Spain

1 ADMINISTRATIVE INFORMATION

1.1 Submission Title: Letter of Intent for biomarkers of drug-induced pancreas injury

1.2 Requesting Information:

Requesting Organization

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1.3 Submission Dates:

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2 INTRODUCTION

There is a critical need for biomarkers of drug-induced pancreas injury (DIPI) to guide safety assessment in phase I clinical trials. Although amylase and lipase, the standard markers of pancreas injury, are currently used in the diagnosis of acute pancreatitis (Working Group IAP/APA Acute Pancreatitis Guidelines, 2013), it is widely acknowledged that they are not sufficiently sensitive or specific to guide dosing-related decisions in clinical trials (Lee and Papachristou 2019; Ismail and Bhayana 2017).

The Innovative Medicines Initiative (IMI) TransBioLine (Translational Safety Biomarker Pipeline) is an IMI2 project with a remit to qualify biomarkers for drug-induced injury of five organ systems. Work Package 3 (WP3) is devoted to drug-induced pancreatic injury (DIPI) and seeks to qualify biomarkers to safely guide dosing in phase I clinical trials involving normal healthy volunteers (NHV) or patients without known pancreatic disease. The envisioned context of use (COU) is for drug candidates where acinar pancreatic injury has been observed in nonclinical toxicology studies or with molecules that represent a known drug class with documented DIPI.

In recent years, extensive work has been undertaken to evaluate improved markers of DIPI in nonclinical species. In addition to protein biomarkers, microRNAs (miRNAs) have received considerable attention owing to the existence of several miRNAs which are exocrine pancreas-specific or enriched (Wong, 2018; Wang, 2018). A recent example is found in a publication by Erdos and colleagues (2020) who compared four miRNAs in plasma to amylase and lipase from 10 rat studies involving multiple pancreatic toxicants. The results showed clear superiority over amylase and lipase for sensitive and specific detection of acinar necrosis as confirmed by histopathology, with miRNA-217 being the best performing biomarker (Erdos, 2020).

An inherent challenge to the qualification of new biomarkers for DIPI is the relatively low clinical incidence of acute pancreatic injury definitively attributable to a drug. Approximately 40 drugs are known to cause DIPI on the basis of case reports of clinical re-challenge (Nitsche, 2012; Conti, 2015); however, even for these agents, DIPI incidence is low and occurrence is difficult to predict. Therefore, WP3 will leverage samples from patients with acute pancreatitis (AP) associated with a variety of causes to investigate potential biomarkers of pancreatic acinar injury. AP is the pathologic consequence of injury to the exocrine pancreas, regardless of etiology, which manifests as a rapid escalation of tissue injury and symptoms following an insult. AP is a necrotizing and inflammatory disorder of the pancreas causing excruciating pain, gastrointestinal dysfunction, and pronounced systemic inflammatory responses with circulatory and respiratory disturbances that can lead to organ failure and death (Forsmark, 2016). While gallstones and excess alcohol consumption account for a majority of cases, the etiology can be multi-factorial, with increasing focus on pharmaceutical drugs as a cause (Bertilsson, 2015; Bazerbachi, 2018; Meczker, 2019; Chung, 2018). Although DIPI has been estimated to account for 2 to 5% of all AP (Jones 2015; Nitsche, 2012), the link between some drugs and AP remains doubtful (Tenner, 2014). The strongest evidence for DIPI is AP that develops during treatment with a drug when no other possible cause can be identified, which resolves upon withdrawal and recurs following reapplication of the drug (Badalov, 2007).

Clinical biomarker analysis by WP3 will occur in two stages. An initial Learning Phase will involve analysis of existing samples collected at clinical presentation from 300 patients with AP

by the Liverpool Pancreas Research Group at the University of Liverpool (ULIV). Biomarkers that differentiate between AP and healthy volunteer values and which exhibit the greatest correlation with AP severity in this learning dataset will be prioritized and taken forward for qualification in a Confirmatory Phase for a defined COU. As detailed herein (Table 7-1), qualification of potential biomarkers will be supported by prospectively collected samples from patients with acute pancreatitis. These samples will be collected in an observational trial referred to as MAP-I (Markers in Acute Pancreatitis) which targets recruitment of as many as 270 patients (70 with DIPI and 200 with AP of other causes). Clinical samples will be collected at three primary sites: University of Liverpool (ULIV); Klinikum der Universität, München, Germany (KUM); and Universidad de Málaga, Spain (UMA). Samples will also be collected from up to 150 normal healthy volunteers (NHV) to be used for comparative analysis (Table 7-1). These samples will be collected at ULIV, as part of MAP-I, and from dedicated clinical trials conducted by two EFPIA members of TransBioLine.

3 DRUG DEVELOPMENT NEED STATEMENT

Currently, drug candidates that cause notable pancreatic acinar cell injury in nonclinical toxicology studies may not progress to clinical testing because of insufficient biomarkers to monitor for the occurrence of DIPI in humans. While exact numbers do not exist to quantify the impact of this phenomenon, there is consensus among the EFPIA representatives in TransBioLine that occurrence of nonclinical exocrine pancreatic injury increases the likelihood for drug attrition. Although the decision to proceed into human testing involves several factors, including the margin of safety, hesitancy clearly stems from the current inability to reliably detect and monitor signals for acinar cell injury in subjects participating in phase I clinical trials.

Biomarkers with improved sensitivity and specificity for detecting exocrine pancreatic injury would decrease the risk of advancing potentially efficacious drugs with nonclinical exocrine pancreatic findings to clinical testing. Consistent with the remit of TransBioLine, WP3 intends to qualify biomarkers for improved safety monitoring in cases where a potential risk for DIPI is identified in nonclinical toxicology studies or where there is a candidate drug with DIPI as a known class effect. Importantly, any biomarker qualified for use in safety monitoring would be deployed in conjunction with pancreatic amylase, lipase and/or clinical monitoring and outcomes currently used to assess pancreatic safety.

4 BIOMARKER INFORMATION AND INTERPRETATION

4.1 Biomarker name

Proteins

The peptides trypsinogen activation peptide (TAP) (PRSS1; [HUGO ID-HGNC:9475]), carboxypeptidase A1 (CBPA1) (CPA1; [HUGO ID- HGNC:2296]) and carboxypeptidase A2

(CBPA2) (CPA2; [HUGO ID- HGNC:2297]), and carboxypeptidase B activation peptide (CAPAP) (CPB1; [HUGO ID HGNC:2299]) have shown promise as biomarkers of pancreatic injury and are known to be elevated during the onset of pancreatitis symptoms (Deng, 2015; Kemik, 2012; Matsugi, 2007; Huang, 2013; Pezzilli, 2000, 2004). A common characteristic to TAP and CAPAP is that each is associated with zymogen activation to yield an active acinar peptidase (trypsin and chymotrypsin B1, respectively). CPA1 and CPA2 are pancreatic zinc proteases that hydrolyze the peptide bond at the C-terminus of amino acid residues. TAP, CPA1, CPA2 and CAPAP will be measured in plasma and urine during the Learning Phase to determine preferred analyte(s) and which matrix to advance to Confirmatory testing. Quantitative analysis will be performed by Signatope, GmbH (Reutlingen, Germany).

TAP is an 8-mer peptide (APFDDDDK) embedded in trypsinogen between an N-terminal signal peptide (1-15) and active trypsin (24-247). It is formed from cleavage by both cationic and anionic trypsinogens (PRSS1 and PRSS2). Because TAP is quickly cleared from plasma, it has historically been measured in urine (Johnson et al, 2004; Huang et al, 2013).

CPA is initially expressed as an inactive zymogen precursor termed pro-carboxypeptidase that is activated upon cleavage by trypsin. The active form of CPA has been shown to be increased in patients with acute pancreatitis (Matsugi, 2007).

CAPAP is an 81-mer peptide (Figure 4-1) formed from cleavage of procarboxypeptidase B1 by trypsin, followed by additional C-terminal proteolysis to yield an 81-mer first identified by Appelros (Appelros, 1998). As CAPAP is much larger than TAP, it has a longer half-life in the circulation prior to urinary excretion. Also, because of its size, CAPAP will be analyzed using a surrogate peptide following cleavage with trypsin. Antibodies have been made to three potential surrogate peptides; whose sequences are underlined in Figure 4-1. Each is currently being evaluated for specificity and recovery.

The exact nature and extent of the C-terminal processing of CAPAP has yet to be fully elucidated. Therefore, as part of this work, a study will be undertaken between ULIV and Signatope using LC/MS/MS to identify CAPAP forms prepared by digestion of recombinant procarboxypeptidase B1 in various pancreas media of human origin. Because it is likely that multiple forms may circulate, we believe that the analytical approach taken may be preferred since it has the potential to simultaneously detect multiple forms differing by their extent of C-terminal truncation. This investigation is also being performed in conjunction with a recognized expert in the field, Miklos Sahin-Toth, MD, PhD (Boston University School of Medicine).

Figure 4-1 CAPAP sequence showing tryptic peptide candidates for immunoprecipitation

```
        10         20         30         40         50         60
HHGGEHFEGE KVFRVNVEDE NHINIIRELA STTQIDFWKP DSVTQIKPHS TVDFRVKAED
        70         80
TVTVENVLKQ NELQYKVLIS N
```

Quantification of the four target enzyme products will be accomplished in both plasma and urine using immunoprecipitation (IP) followed by nano-liquid chromatography tandem mass spectrometry (IP-LC-MS/MS). The antibodies used for IP will specifically target surrogate peptides produced from trypsin digestion of the sample. An exception is TAP, which is pulled down by an antibody that targets the intact 8-mer. To account for differences in digestion recovery, standard curves will be prepared using recombinant standards for CPA1, CPA2 and CAPAP. A synthetic peptide standard will be used for TAP. Details about the method can be found in Attachment 11.1.1.4 and Attachment 11.1.1.5.

Micro-RNA

RNA sequencing and analysis will be conducted by two consortium partners: TAmiRNA, GmbH (Vienna, Austria) and the Functional Genomics Center Zurich (FGCZ), University of Zurich. MicroRNAs are small non-coding RNA molecules of approximately 22 nucleotides in length that control gene expression in all mammalian cells. A diverse array of functions including cellular signaling, cell growth and differentiation and apoptosis have been described for miRNAs. In humans, approximately 2600 miRNA genes are currently known. Information on miRNA identification and nomenclature can be found at www.mirbase.org. Because of the relative abundance of miRNAs in specific tissues, they are increasingly being recognized as leakage biomarkers indicative of tissue injury (Bailey, 2018; Schraml, 2017). Although a number of candidate miRNAs have been identified and studied in association with pancreatic acinar injury in nonclinical species (Erdos, 2020; Usborne, 2014), these will not automatically be advanced for confirmatory analysis. Rather, next generation sequencing (NGS) will be used to profile the whole transcriptome of miRNAs present in the same samples used for exploratory protein analysis. A prioritized list of the best performing miRNA candidates identified by exploratory analysis will be advanced for confirmatory testing using RT-qPCR.

4.2 Analytical methods

Novel Peptide/Protein Biomarkers

TAP, CPA1, CPA2 and CAPAP will be measured in EDTA-plasma and urine using IP-LC-MS/MS with peptide immunoaffinity capture at Signatope. The IP-LC-MS/MS methodology deployed has been previously published (Weiß, 2015). Proteins will be measured in EDTA-plasma and urine using IP-LC-MS/MS with peptide immunoaffinity capture. Nano LC-MS/MS analysis will be performed on a TQ6500+ system (ABSciex) by multiple-reaction monitoring (MRM). Analytical validation will occur in two stages. Learning Phase analysis of candidate biomarkers will occur using a method validated by a fit-for-purpose strategy. Protein(s) taken for subsequent confirmation will undergo full validation pursuant to the FDA Bioanalytical Method Validation Guidance for Industry (May 2018) and informed by the recent publication (Piccoli, 2019).

Standard Protein Biomarkers

Each subject in the Confirmatory Phase will have serum/plasma biomarkers measured using existing methods validated for clinical study at MLM Medical Labs, GmbH (Mönchengladbach, Germany). The specific biomarkers are as follows: pancreatic amylase, lipase, interleukin-6 (IL-6), and C-reactive protein (CRP). Because these biomarkers are currently used to diagnose pancreatitis, they provide an important comparison point from which to judge the performance(s) of novel markers. The biomarkers will be analyzed in serum using a Cobas 6000 system from Roche diagnostics. For pancreatic amylase and lipase enzymatic methods are used. CRP is analyzed using a turbidimetric assay and IL-6 an electrochemiluminescence assay (ECLIA) assay.

MicroRNA

For identification of miRNAs, exploratory analysis will occur using a fit-for-purpose validated Next Generation Sequencing (NGS) platform designed to survey the entire miRNA transcriptome in plasma and urine samples from healthy volunteers and patients with AP. This method provides unbiased, genome-wide quantitative (i.e. copy number) analysis of circulating miRNAs using a small volume (200 µl). Standard Operating Procedures (SOPs) will be followed which describe procedures for collecting and storing EDTA plasma including details such as types of collection tubes to use, incubation times, centrifugation parameters, and storage conditions. Quality control will include assessment for hemolysis (spectrophotometric analysis) and enzyme inhibition (RT-qPCR of spike-in controls). Confirmatory analysis of select miRNAs will be performed by RT-qPCR, the preferred choice for targeted bioanalysis of miRNAs in human biofluids (Mestdagh, 2014). Both NGS and RT-qPCR have demonstrated cross-species comparability among mammals which will enable nonclinical confirmation of biomarker performance. The analytical fit-for-purpose validation plan for NGS includes accuracy (spike-in recovery), precision, the analytical measurement range (LLOQ, ULOQ), and multiplexed sequencing validation. The analytical

validation plan for RT-qPCR includes accuracy (spike-in recovery and amplification efficiency), precision (repeatability and reproducibility), the analytical measurement range (LLOQ, ULOQ), parallelism (dilution linearity), sensitivity, and stability.

4.3 Measurement units and limit(s) of detection

Protein biomarkers in plasma will be expressed in terms of concentration (e.g., pg/mL or nM). For urine, protein concentrations will be normalized to urine creatinine concentration in order to account for differences in urine volume. The detection limits for TAP, CPA1, CPA2 and CAPAP are not known at this time. Quantitative analysis of miRNAs in confirmatory plasma or urine samples will occur by RT-qPCR. The output will be expressed as quantification cycle (Cq) with a limit of detection at Cq = 40. normalized Cq/Ct-values. MiRNAs with non-human sequences will be used as internal standards for normalization of RT-qPCR data as recently described (Starlinger, 2019).

4.4 Sample handling, shipment and storage

Exploratory samples were obtained from AP patients at the University of Liverpool (ULIV) under existing protocols. Urine samples were collected at presentation, whereas plasma was collected longitudinally over multiple timepoints. All samples have been stored at -80^{o}C. Prospective samples will be collected and stored using procedures developed by the TransBioLine consortium.

The Central Biobank of the Charité (Berlin, Germany; ZeBanC) is responsible for prospective sample and ID management. A harmonized SOP and instructions for sample handling and shipment will be established which will then be used by all recruitment sites. Furthermore, pre-designed kits for structured acquisition of biomaterials including color-coded 2D barcoded tubes (FluidX/ Brooks) are provided.

These sample kits come with sample-related data to document standard sample annotation information (e.g. date and time of sampling, volume, duration until processing, centrifugation conditions). This is of utmost importance to assess the quality of the collected biomaterials for biomarker analysis in our consortium. The standard preanalytical coding for biospecimen (SPREC) facilitates the documentation of preanalytical quality parameters used in our biobank and will provide consistent data. The recruitment centers will organize shipment. After notification the aliquots will be sent on dry ice with temperature monitoring.

In the ZeBanC the samples will be stored in monitored deep-freezing systems. Aliquot data and location are documented in the ZeBanC LIMS system and are traceable at any time.

4.5 Biomarker interpretation and utility

A fundamental premise of this work is that exocrine pancreas-specific biomarkers can be identified, and that they will demonstrate a discernible pattern prior to or during the onset of

symptoms resulting from the necrosis of pancreatic acinar cells. However, until we have completed the Learning Phase analyses, the exact biomarker candidates will be unknown. Both individual and composite measures will be examined for their ability to differentiate NHV subjects from patients with pancreatitis and the biomarkers/approach showing the best performance will be interrogated in the Confirmatory Phase. Ultimately, selection of individual biomarker(s) versus a composite measure will depend on the observed effect size compared to established reference intervals and the ability to classify observed clinical outcomes. Standard performance measures such as positive and negative predictive value (PPV/NPV) will be used in this determination. Another question that will be investigated is whether the novel biomarker(s) can be reliably compared to a baseline (pre-treatment) value for each subject in a phase I clinical trial or whether it will be necessary to compare the mean/median values from cohorts of individuals. Regardless of the path taken, the novel biomarker(s) will be evaluated against and used in conjunction with existing markers and safety measures that are the standard of care to diagnose and assess pancreatitis.

5 CONTEXT OF USE STATEMENT

A safety biomarker or biomarker panel to aid in the detection of acute acinar pancreas injury in phase I trials where there is an *a priori* concern that a novel drug may induce pancreas injury (DIPI) in humans.

6 ANALYTICAL CONSIDERATIONS

6.1 Pre-analytical Considerations

Pre-analytical factors related to clinical sample collection and storage will be evaluated during the development and validation of all methods for both miRNAs and proteins. While long-term storage stability of miRNAs remains to be established, a recent publication on pre-analytical and storage conditions confirmed viable long-term storage for miRNAs in plasma (Glinge, 2017). This finding was supported by a more recent publication (Matias-Garcia, 2020).

Learning Phase samples were acquired and stored under SOPs established at ULIV. Plasma and urine have been stored at -80^{o}C since collection and processing. Samples from AP patients were acquired continuously over a period beginning in 2010.

6.2 Analytical Considerations

All assay validations in the Learning and Confirmatory Phases will be conducted using a fit-for-purpose approach. In the Learning Phase, miRNA will be measured using a recently validated NGS method (Attachment 11.1.1.2). In the Confirmatory Phase a select set of individual miRNA molecules will be quantified by RT-qPCR. The validation plan for this method appears as Attachment 11.1.1.3. As many as six miRNAs may be advanced for qualification.

The protein 4-plex will (TAP, CPA1, CPA2 and CAPAP) with be measured by IP-LC-MS/MS using the methods described in Attachment 11.1.1.4 (plasma) and Attachment 11.1.1.5 (urine). These methods are currently being validated for use in the Learning Phase according the validation plans appearing as Attachment 11.1.1.6 (plasma) and Attachment 11.1.1.7 (urine). Our current plan calls for taking a single protein and matrix forward for qualification in the Confirmatory Phase. A dedicated validation of the associated method will be conducted with the full rigor expected for biomarker qualification (Piccoli, 2019).

7 CLINICAL CONSIDERATIONS

7.1 Use in Drug Development

The qualified DIPI biomarker will be applied in conjunction with the totality of information derived from existing biomarkers and clinical observations to guide dosing decisions in the phase I clinical setting. A proposed decision tree is provided in Figure 7-1. As shown, the biomarker will be applied in cases where there is an *a priori* concern of drug-induced acinar pancreas injury. Examples could include the observation of acinar damage in one or more species by histopathology during nonclinical toxicology studies or cases where a drug shares structural similarity to a compound known to be associated with DIPI. When such conditions exist, the biomarker or composite panel would be measured prior to drug administration to establish a baseline for the subject. The primary application will be to normal human volunteers (NHV) in phase I clinical trials, but we believe application to additional select patient populations (e.g. cancer patients) with no known pancreatic disease may also be appropriate.

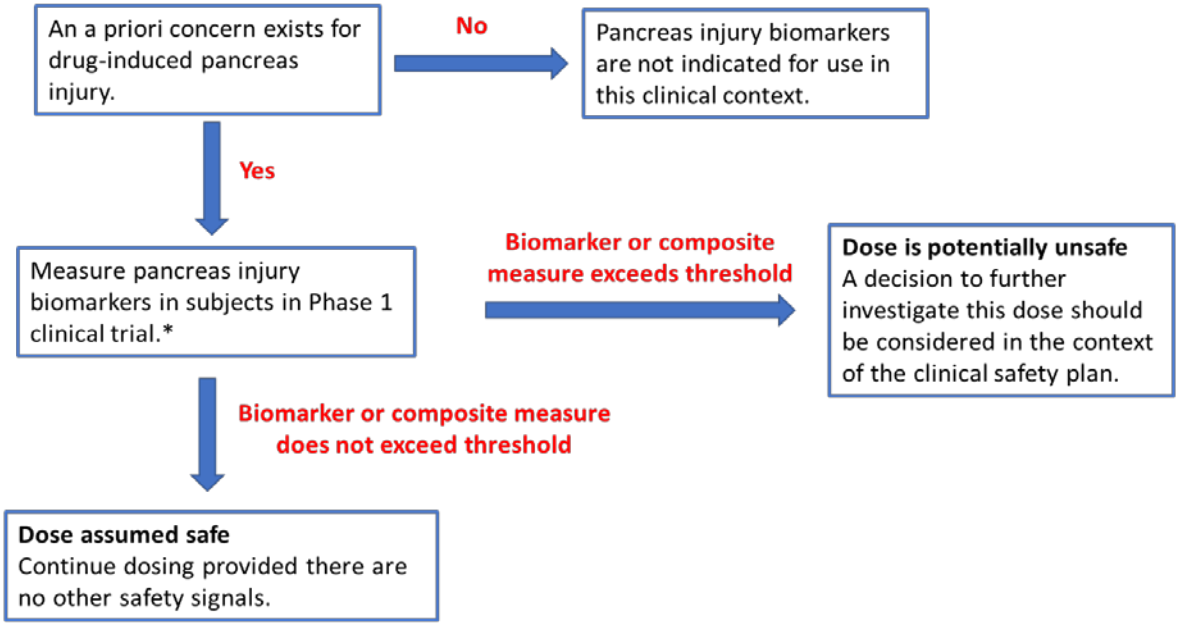
The basis for clinical decision making (Figure 7-1) is an elevation of the biomarker or composite measure above a pre-determined threshold. The nature of this threshold has not been defined and awaits data from the Learning Phase. The proposed approach will ultimately be tested through Confirmatory analysis. Regardless of the method used, a baseline sample will be obtained from all subjects prior to dose administration. Although reference intervals will be established for the qualified biomarker(s), it is possible that statistical power may not exist for decision-making to be made relative to a threshold derived from these intervals (e.g. fold-change versus the upper

limit of normal). In such a scenario, the threshold would be established as a fold-change relative to the subject's individual baseline, or the mean/median baseline value for the trial cohort.

In cases where the biomarker signal does not exceed the threshold, a presumption would be made that no injury is present. Importantly, a decision to proceed would be made alongside standard clinical measures including amylase and lipase. Further, the decision to continue to apply the biomarker following a negative result would be made by the trial sponsor.

In cases where the biomarker signal is elevated above the defined threshold, a presumption would be made that acinar damage had occurred and the dose would therefore be considered unsafe. A decision to continue dosing, for example following dose-adjustment, would be made by the sponsor in accordance with a plan for safety monitoring that would include continued measurement of the qualified biomarker(s).

Figure 7-1. Decision tree for clinical use of exocrine pancreatic-injury biomarkers during novel drug development



*Signal will be compared to pre-established reference interval or baseline measurement.

7.2 Patient population or drug development setting

The qualified biomarker will be applied to NHV subjects or patients without known pancreas disease in phase I clinical trials. Apart from pancreas disease, no exclusion criteria are envisioned at this time; however, this matter is subject to change as more is learned about the biomarkers in the clinical setting.

The clinical use of the biomarkers is envisioned as a supplement to standard methods to diagnose pancreatitis (e.g. amylase, lipase, clinical symptoms, imaging) and to potentially offer more sensitive and specific measures to detect and monitor acinar cell degeneration/necrosis.

7.3 Clinical validation

7.3.1 Learning Phase evaluation of potential DIPI biomarker panel

In the Learning Phase, we will measure candidate biomarkers in archived samples from 300 patients presenting at ULIV and diagnosed with AP. Each of the AP patients was followed for a period of at least one month allowing the patients outcome to be categorized as mild (n=150), moderate (n=100) or severe (n=50) according to the Revised Atlanta Criteria (Banks, et al. 2013). Each of the samples to be analyzed were acquired at the time of clinical presentation. Plasma was obtained from all patients, while urine was collected from a subset of 60 patients. Analysis of this retrospective sample set will characterize biomarker levels in patients relative to the severity of AP. Candidate biomarkers will also be measured in samples prospectively collected from NHVs acquired in Study H60-MC-O014, (see Section 7.3.3) to characterize expected variability. The goal of the Learning Phase is to prioritize which DIPI biomarkers (e.g. a small set of miRNAs in addition to a single protein biomarker candidate) will be studied in the Confirmatory Phase. Prioritization will be guided by the magnitude of change of candidate biomarkers in AP patients relative to NHV subjects, the correlation of candidate biomarkers to severity of AP, and observed variability of candidate biomarkers.

7.3.2 Confirmatory Phase evaluation of potential DIPI biomarker panel

Biomarker candidates (either individual biomarkers or a composite measure) prioritized from the Learning Phase analysis will be evaluated in the Confirmatory Phase. A key objective to support qualification is to establish a threshold for biomarker change that reliably differentiates clinically diagnosed pancreatitis from the upper limit of normal in NHV subjects. Table 7-1 summarizes the source and recruitment targets for samples that will support qualification. As indicated, the majority of samples will come from patients with AP recruited into the observational study MAP-I. Patients will be followed longitudinally to allow their outcome status to be categorized as mild, moderate or severe using the Revised Atlanta Criteria. The study aims to collect samples from a total of 270 patients presenting with AP, which includes 70 patients that are receiving a drug that is associated with DIPI at the time of diagnosis. A comprehensive list of 93 drugs associated with AP has been compiled through expert review of the scientific literature that includes clinical case reports with re-challenge, mechanistic studies, case series, clinical trials, systematic reviews and epidemiologic data. This list will be used to identify AP patients that will be further categorized as DIPI. Plasma and urine samples will be collected at presentation from all patients and will serve as the foundation for biomarker performance. Additional plasma sample will be collected

on days 4 and 14 from all patients at ULIV and may be collected at other sites. These longitudinal samples will allow further characterization of biomarker changes over time in AP.

Qualification of candidate biomarkers will be supplemented by data demonstrating the specificity of the biomarkers for acute pancreatic injury. Samples will be collected from contrast groups representing patients with other forms of pancreatic injury/disease: chronic pancreatitis, diabetes mellitus, and pancreatic ductal adenocarcinoma. These samples will provide key information about the specificity of the candidate DIPI biomarkers to detect acute pancreatitis relative to more chronic conditions. In addition, specificity for the pancreas relative to other organ system disease/injury will be assessed by analyzing candidate pancreatic biomarkers in samples from the other work packages in TransBioLine (i.e. kidney, liver, vascular and neuro).

It is acknowledged that biomarker signals could be observed in the contrast groups and that there may be insufficient statistical power to show specificity. Nevertheless, the information obtained will be valuable to understanding the application and interpretation of any qualified biomarker(s) and will guide future investigation.

7.3.3 Reference Interval Determination

Table 7-1 summarizes the NHV subjects that will be prospectively recruited to support qualification. Healthy volunteer samples will be collected at the ULIV and from dedicated studies conducted by two consortium partners (Eli Lilly and Roche). Study H60-MC-O014 (Prospective Evaluation of Novel Biomarkers of Drug-induced Acute Pancreatitis in Healthy Subjects) was sponsored by Eli Lilly and completed at Covance (Leeds, UK) in January 2020. In this study, three (3) plasma samples and a single urine collection were obtained from 60 participants. The plasma samples include collections from fasted and post-prandial states. Samples from study H60-MC-O014 will support the Learning Phase evaluation (Section 7.3.1). Reference interval determination in the Confirmatory Phase will occur using NHV samples to be collected from ULIV along with a Roche-sponsored study.

Clinical and Laboratory Standards Institute (CLSI) guidance EP28-A3c will be used to guide reference interval determination for each individual or composite biomarker taken for qualification (Horowitz, 2014). Reference intervals will be determined in both plasma and/or urine based on the biomarkers from the Learning Phase that are chosen to be advanced to confirmatory analysis. The reference intervals will be established from analysis of samples from at least 100 healthy volunteers (maximum 150) recruited to achieve a balance in age and gender. Both fasted and post-prandial samples will be obtained to investigate the effect of post-prandial responses in exocrine pancreas function on candidate biomarker variability.

7.3.4 Statistical Analysis Plan

The objective of the Statistical Analysis Plan (SAP) is to describe a statistical strategy for the validation of the biomarkers under consideration for acute pancreatic injury. The assessment will be done by considering reliability and validity of each biomarker individually and as a composite set. The goal is to identify the panel of biomarkers that consistently detect changes due to acute AP by comparing acute AP patients and NHVs, and to quantify the sensitivity and specificity by comparing acute AP patients and patients with other diseases.

Learning phase evaluation of potential DIPI biomarkers

In the learning phase, the statistical analysis objective is to evaluate a large panel of potential biomarkers of their capacity to detect AP status and then select a small set of biomarkers for further analysis in the confirmatory phase. The potential pancreatic injury protein biomarkers (TAP, CPA1, CPA2 and CAPAP) will be tested in either plasma or urine together with current standard markers (amylase, lipase, IL-6 and CRP) in about 60 NHVs from Lilly trial and 300 acute AP patients from ULIV research tissue Biobank (150 mild, 100 moderate and 50 severe acute AP) with samples collected at presentation. In addition, miRNA expression data from RNAseq will be evaluated.

Based on data availability, data quality and their ability to detect the difference between NHVs and different disease status, a preferred matrix will be selected for further analysis. For each biomarker, the data will be fit to an ANOVA model including disease status, age, gender, race, and other covariates if available and deemed important to quantify biomarker expression. Model assumptions - data normality and variance homogeneity will be checked. The data may be log-transformed or non-parametric analysis methods may be applied if necessary. The false discovery rate (FDR) approach will be applied to test p-values for multiple testing adjustment. However, for biomarkers with prior evidence of correlation with AP, i.e., protein biomarkers and some reported miRNAs, the significance may be evaluated on raw p-values instead of FDRs.

A small set of protein and miRNA biomarkers will be selected for further analysis based on combined evidences including the following:

1. Significance testing over different disease severity groups and NHVs.
2. Fold change comparison over different disease severity groups and NHVs, and if the difference is clinically/biologically meaningful.
3. Correlation between biomarkers. If several biomarkers are highly correlated, only one may be selected since they will present similar capacity detecting AP disease status.
4. Functional relevance and pathway analysis.

After a small set of biomarkers have been selected, multivariate analysis may be performed to further refine the selection and establish a final biomarker panel and explore composite score approach to be tested/validated in the confirmatory phase.

Confirmatory phase evaluation of potential DIPI biomarkers

In the Confirmatory Phase, selected biomarkers from the Learning Phase will be qualified in new, more diverse patient cohorts. Protein biomarkers are to be measured with the same analytical method as in the learning phase, miRNA biomarkers are to be measured using a different assay, RT-qPCR. The patient samples include about 150 NHVs from two trials, 70 DIPIs, 200 other cause AP (100 mild, 50 moderate, 50 severe), 25 chronic pancreatitis, 25 pancreatic cancer, 25 type1 or type 2 diabetes.

All statistical procedures described in the Learning Phase, correlation, statistical comparison and multivariate analysis are applicable for the Confirmatory Phase. The statistical analysis outlined in the Learning Phase, will be applied on all biomarkers that are selected for the Confirmatory Phase. For the selected miRNAs, samples from the Learning Phase will also be assayed with RT-qPCR to bridge between RNAseq and RT-qPCR data. DIPIs and other cause APs may be combined if they do not show substantial difference considering the small sample size in the DIPI cohort. By comparing acute AP with NHVs and other disease patients, the performance of the DIPI biomarker will be quantified.

After the data analysis is completed for the Learning Phase and the results are interpreted, a detailed SAP for the Confirmatory Phase will be generated and will be available prior to generating confirmatory data used for qualification.

Table 7-1 Samples to support Confirmatory Phase of DIPI biomarker qualification

Objective	Study	Site/Sponsor	Number of Subjects	Samples/Subject (Matrix)
Acute Pancreatitis (AP) *Any etiology* Evaluate candidate biomarkers in plasma and urine from AP patients	MAP-I	ULIV KUM UMA	200[a]	1-3 (plasma[b]) & 1 (urine)
Suspect Drug-Induced Pancreatic Injury (DIPI) Evaluate candidate biomarkers in AP patients taking drugs associated with DIPI	MAP-I	ULIV KUM UMA	70	1-3 (plasma[b]) & 1 (urine)
Contrast Groups Measure candidate biomarkers in patients with chronic pancreatic diseases[c] to understand specificity of biomarkers for acute pancreatitis	MAP-I	ULIV KUM UMA	75[c]	1 (plasma) & 1 (urine)
Measure candidate biomarkers in patients with diseases/injury of other organ systems[d] to understand specificity of biomarkers for pancreas injury	TransBioLine	Other consortium work packages	80[d]	1 (plasma)
Normal Healthy Volunteers (NHV) Establish reference ranges of candidate biomarkers in plasma and urine of healthy volunteers	MAP-I	ULIV	100	1 (plasma) & 1 (urine)
	H60-MC-O014	Covance-Leeds/ Lilly	60	3 (plasma[e]) & 1 (urine)
	TBD	Roche	50	3 (plasma[e])

[a] Longitudinal assessment will categorize severity of AP as Mild (n=100 patients), Moderate (n=50 patients), or Severe (n=50 patients)

[b] Plasma and urine will be collected at clinical presentation from every patient. Additional plasma samples will be collected on days 4 and 14 (Total of 3 samples) from every patient recruited at ULIV and is optional from patients recruited at KUM and UMA

[c] Chronic pancreatitis (n=25 patients), Diabetes mellitus (n=25 patients), Pancreatic ductal adenocarcinoma (n=25 patients)

[d] Kidney, liver, vascular, and neurological disease/injury (n=20 patients/organ system)

[e] Includes sampling in a fasted and post-prandial state

7.4 Benefits and risks

For drug development, the primary benefit to improved clinical biomarkers for DIPI is to advance potentially efficacious therapies into clinical trials that might otherwise be stopped prematurely because of the inability to safely monitor for pancreatic injury using existing biomarkers.

From a safety perspective, the greatest risk stems from false negative results, as this would put patients at risk. Despite the opportunity to improve upon amylase and lipase, it is essential that the qualified biomarker(s) have sufficient performance as determined by NPV to permit the safe testing of new chemical entities in phase I trials that have shown nonclinical acinar cell injury or are part of a drug class with documented DIPI.

While false positives present less risk to trial participants, it increases the risk of unwarranted termination of promising drug candidates. It will be important to understand sources of interference or variability that could confound the observed signal. Accordingly, samples from other target organ injury groups collected within the TransBioLine consortium will be investigated to demonstrate specificity of the novel biomarker(s).

7.5 Current knowledge gaps, limitations, and assumptions

The fact that select miRNAs are abundant and preferentially expressed in the pancreas affords an opportunity to qualify biomarkers of acinar pancreas necrosis/degeneration that can out-perform amylase and lipase. Despite the excitement around this opportunity, there is much to be learned about the performance and biology associated with novel biomarkers for DIPI.

Several questions remain to be answered concerning the type of acinar injury represented by the biomarkers studied as well as their ability to translate from nonclinical species to the clinic. Nonclinical studies involving the qualified biomarker(s) will be performed in conjunction with histopathology to address this question. Another source of uncertainty surrounds the validity of using all causes of acute pancreatitis as a legitimate surrogate for DIPI. Although this approach is necessary from a practical standpoint, extended clinical study will be required to understand the accuracy of this assumption. In addition to these factors, a recognized gap comes from the fact that the MAP-I study will only be able to recruit patients that are symptomatic and likely have elevated amylase and lipase. Therefore, the ability to detect acinar pancreas damage prior to the presentation of symptoms is uncertain. A comparison between the novel biomarker(s) and histopathology in nonclinical studies will be used to explore whether a premonitory window exists in the species being studied.

8 SUPPORTING INFORMATION

8.1 Association of biological processes with proposed biomarkers

The proposed biomarkers (miRNA and protein) are released from the pancreas into plasma or urine following acinar cell degeneration/necrosis. Four miRNA biomarker candidates (miR-216a, miR-216b, miR-217, and miR-375) were evaluated in plasma from 20 rat sensitivity and specificity studies and compared with the amylase and lipase enzymatic assays to determine their relative performance in monitoring for drug-induced pancreas injury (Erdos et al., 2020). Circulating levels of these pancreas-specific miRNAs increase over baseline in response to various pancreatic toxicants and correlate with the severity of acinar cell injury as determined by histopathology. The biomarkers also remained elevated longer relative to amylase and lipase which could allow a larger window of time to detect clinical AP. This work provides a proof of concept that miRNA molecules can add value in assessing DIPI.

In the pancreas, trypsinogen activation peptide (TAP) is cleaved from trypsinogen through enterokinase resulting in trypsin which in turn activates digestive pancreatic proenzymes in the duodenum. Nonclinical assessment of TAP has demonstrated an association with AP in humanized mouse models with mutant forms of PRSS1 (R122H and N29I); the most commonly mutated gene in human hereditary pancreatitis (Athwal et al, 2014; Gui et al, 2020). The underlying mechanism of pancreatitis is thought to involve the premature activation of trypsinogen in the acinar cells. This “gain-of-function” mutation in humans results in a trypsin-mediated trypsinogen autoactivation. These studies demonstrate human PRSS1 expressed in mice predisposes these animals to AP and increases susceptibility to cerulein induced pancreatitis. Staubli et al. (2015) reviewed the clinical literature and described multiple studies where TAP has been assessed in urine and serum and found to be elevated with AP. Similarly, the proenzyme carboxypeptidase B is activated by trypsin through cleavage of carboxypeptidase activation peptide B (CAPAP). Currently there is no published information on CAPAP being evaluated for toxicity assessment in animal models of DIPI, there are however, a number of clinical studies that have shown a good correlation between CAPAP increases and severity of AP (Staubli 2015).

A recent study describes the in vivo association of a human CPA1 mutation and misfolding of the protein resulting in chronic pancreatitis in mice where the most frequently occurring human mutation was knocked-in (Hegyi and Sahin-Toth, 2019).

Collectively the evidence demonstrates that these proteins are critical for normal pancreatic function. The assumption is that pancreatic acini leakage biomarkers correlate to the same histopathology endpoint in humans irrespective of mechanism of toxicity or disease pathogenesis as defined by standard endpoints of imaging, functional tests, established circulating biomarkers, and/or standard clinical chemistry tests.

8.2 Nonclinical assessment and plans for consideration

We plan to study nonclinical models of drug-induced pancreatic injury to generate supportive data for the qualification of biomarkers of DIPI for phase I clinical trial testing. Proposed biomarkers are conserved across species, and analytical assays for rodents are available for most of the proposed biomarkers. Nonclinical evaluation will serve to correlate histopathological changes directly to biomarker responses both in magnitude and duration as well as in assessing reversibility of injury. This type of detailed biomarker characterization cannot be achieved clinically due to limited patient sampling and the narrow breadth of compounds represented in that population. Nonclinical data also allows for an assessment of a biomarker's specificity to DIPI rather than other target organ toxicities (e.g., liver or skeletal muscle injury). However, our intent is that the nonclinical data will serve as supporting information rather than the primary basis for the final qualification decision and the proposed COU. IMI WP3 collaborators have committed to conducting nonclinical studies to ensure that multiple compounds are evaluated.

9 PREVIOUS QUALIFICATION INTERACTIONS AND OTHER APPROVALS (IF APPLICABLE)

On March 10, 2020 a Pre-LOI meeting was held with the FDA to discuss a dual COU plan involving both safety and prognostic endpoints. At this meeting the FDA indicated that these COUs must be qualified separately. The present document, which focuses on a safety biomarker, reflects this feedback.

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11 ATTACHMENTS

11.1 Other supporting information (*Optional – not for public posting*)

The following optional confidential information is being submitted as optional attachments and should not be publicly posted.

11.1.1 Analytical method validation

11.1.1.1 Next Generation Sequencing Method Validation Work Plan

11.1.1.2 Next Generation Sequencing Method Validation Report

11.1.1.3 RT-qPCR Validation Plan

11.1.1.4 IP-LC-MS/MS method description – plasma

11.1.1.5 IP-LC-MS/MS method description – urine

11.1.1.6 IP-LC-MS/MS Method Validation Plan for TAP, CPA1, CPA2 and CAPAP in human plasma

11.1.1.7 IP-LC-MS/MS Method Validation Plan for TAP, CPA1, CPA2 and CAPAP in human urine