1 Executive Summary

1.1 Administrative Overview

Requestor name, title(s) & contact information

John-Michael Sauer, Ph.D.
Critical Path Institute
1730 E. River Rd.
Tucson, AZ 85718
Email: jsauer@c-path.org
Phone: 520-382-1396

Alternate requestor name, title(s) & contact information

Nicholas King
Critical Path Institute
1730 E. River Rd.
Tucson, AZ 85718
Email: nking@c-path.org
Phone: 520-777-2881

Collaboration: name of supporting or participating organization, consortia or individuals.

Critical Path Institute (C-Path) Predictive Safety Testing Consortium's (PSTC) Hepatocyte Working Group (HWG)

Project title

Qualification Plan for Glutamate Dehydrogenase (GLDH) as a Biomarker of Drug Induced Liver Injury in Individuals with Skeletal Muscle Degeneration

History

- *Submitted Letter of Intent to FDA – November 2016*
- *Submitted Briefing Book to FDA and EMA – December 2016*
- *Held joint meeting with FDA and EMA on Briefing Book – March 2017*
- *Responded to questions and issues from FDA and EMA – Q2-3 2017*
- *Received Letter of Support for GLDH from EMA – November 2017*
- *Submitted Legacy Biomarker Qualification Project Status Update to FDA – May 2018*
- *Received Decision Letter on Legacy Biomarker Qualification Project Status Update from FDA – October 2018*

1.2 Background

Drug-induced liver injury (DILI) remains the single greatest cause for termination of development of drug candidates and withdrawal of approved drugs from the market (Yuan and Kaplowitz, 2013; Kaplowitz, 2005). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum

activity measurements are used as current standard biomarkers for the identification of liver injury in clinical practice, and commonly used to assess risk of liver injury during drug development. Hy's Law and a recent definition by an international DILI Expert Working Group (EWG) provide guidance to help differentiate mild or transient hepatocellular injury from more severe DILI and functional hepatocellular damage (Temple, 2006; FDA, 2009; Aithal et al., 2011). A 3x and 5x ALT elevation from the upper limit of normal (ULN) are commonly used thresholds of concern in clinical trials, triggering confirmatory testing and close observation of an individual as suggested in the Food and Drug Administration (FDA) Guidance for Industry: Drug-Induced Liver Injury: Premarketing Clinical Evaluation (FDA, 2009). However, increases in ALT do not always signal hepatocellular injury.

Although ALT and AST are sensitive markers of hepatocellular injury, both enzymes are also expressed in other tissues such as muscle. This severely limits the utility of ALT and AST as markers of liver damage in subjects with underlying muscle impairments, such as Duchenne muscular dystrophy (DMD) or other neuromuscular diseases, in clinical trials with simultaneous drug-induced liver and muscle injury or even in subjects engaging in strenuous exercise. In addition, increased levels of ALT and AST due to underlying muscle damage may potentially mask a hepatotoxic signal, creating a diagnostic challenge for clinicians. Therefore, the development of additional biomarkers of DILI is warranted.

Glutamate Dehydrogenase (GLDH) is a mitochondrial enzyme that plays a role in amino acid oxidation and urea production. GLDH is primarily found in the liver with only a trace amount in skeletal muscle (Mastorodemos et al., 2005; Jaeschke and McGill, 2013). In humans, serum GLDH activity is elevated in patients with hepatic ischemia (Kretzschmar et al., 2003), progressively increasing with increased severity of disease (Schmidt and Schmidt, 1988). Furthermore, serum GLDH activity has been shown to be a sensitive marker of a mild hepatocyte necrosis in patients treated with heparin (Harrill et al., 2012). Like ALT, increases in GLDH within 8 hours of acetaminophen (APAP) overdose predicted patients that proceeded to acute liver injury (Antoine et al., 2013). GLDH has a half-life of 18 hours (Schmidt and Schmidt, 1988; Schmidt and Otto, 1967) whereas ALT has a half-life of 47 hours (Kim et al., 2008; Giannini, 2005), thus GLDH levels would be expected to return to baseline more quickly than ALT levels upon resolution of injury. However, unlike ALT and AST, GLDH activity does not increase following muscle injury or degeneration (Thulin et al., 2014).

As part of the Critical Path Institute (C-Path) Predictive Safety Testing Consortium's (PSTC) ongoing efforts to augment translational biomarker tools for DILI, the Hepatotoxicity Working Group (HWG) is proposing the qualification of serum GLDH activity as a biomarker of liver injury to confer specificity to the liver in human subjects with underlying muscle injury or degeneration. Serum GLDH is a safety biomarker capable of detecting drug-induced hepatocellular injury that can be used in place of ALT in clinical trials for subjects and patients with elevated serum

transaminases due to muscle injury or degeneration and should be used in conjunction with standard hepatic injury monitoring biomarkers, alkaline phosphatase (ALP) and total bilirubin (TBil).

1.3 Biomarker Name, Type and Description

Glutamate dehydrogenase (GLDH), a mitochondrial enzyme expressed primarily in liver, is a molecular biomarker whose activity can be measured in serum. The unique identifier for GLDH is UniProt ID P00367. GLDH is a safety biomarker capable of sensitively and specifically detecting drug-induced hepatocellular injury in humans and nonclinical species.

1.4 Context of Use (COU) Statement

Serum glutamate dehydrogenase (GLDH) is a safety biomarker capable of detecting drug-induced hepatocellular injury that can be used in place of alanine aminotransferase (ALT) in clinical trials for subjects and patients with elevated serum transaminases due to muscle injury or degeneration. GLDH should be used in conjunction with standard hepatic injury monitoring biomarkers (e.g. total bilirubin and alkaline phosphatase).

The current COU has evolved beyond its previous iteration, narrowing its focus to the utility of GLDH in subjects and patients with muscle injury and degeneration.

1.5 Measurement Method

GLDH can be reliably measured in serum. The Randox GLDH assay utilizes the conversion of α-oxoglutarate to glutamate for detection of GLDH enzymatic activity as recommended by the Deutsche Gesellschaft für Klinische Chemie (DGKC).

$$\alpha - \text{oxoglutarate} + \text{NADH} + \text{NH}_4^+ \xrightarrow{\text{GLDH}} \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

In this reaction, the kinetics of NADH oxidation are proportional to the GLDH activity and measured spectrophotometrically as a decrease in absorbance per minute at 340 nm.

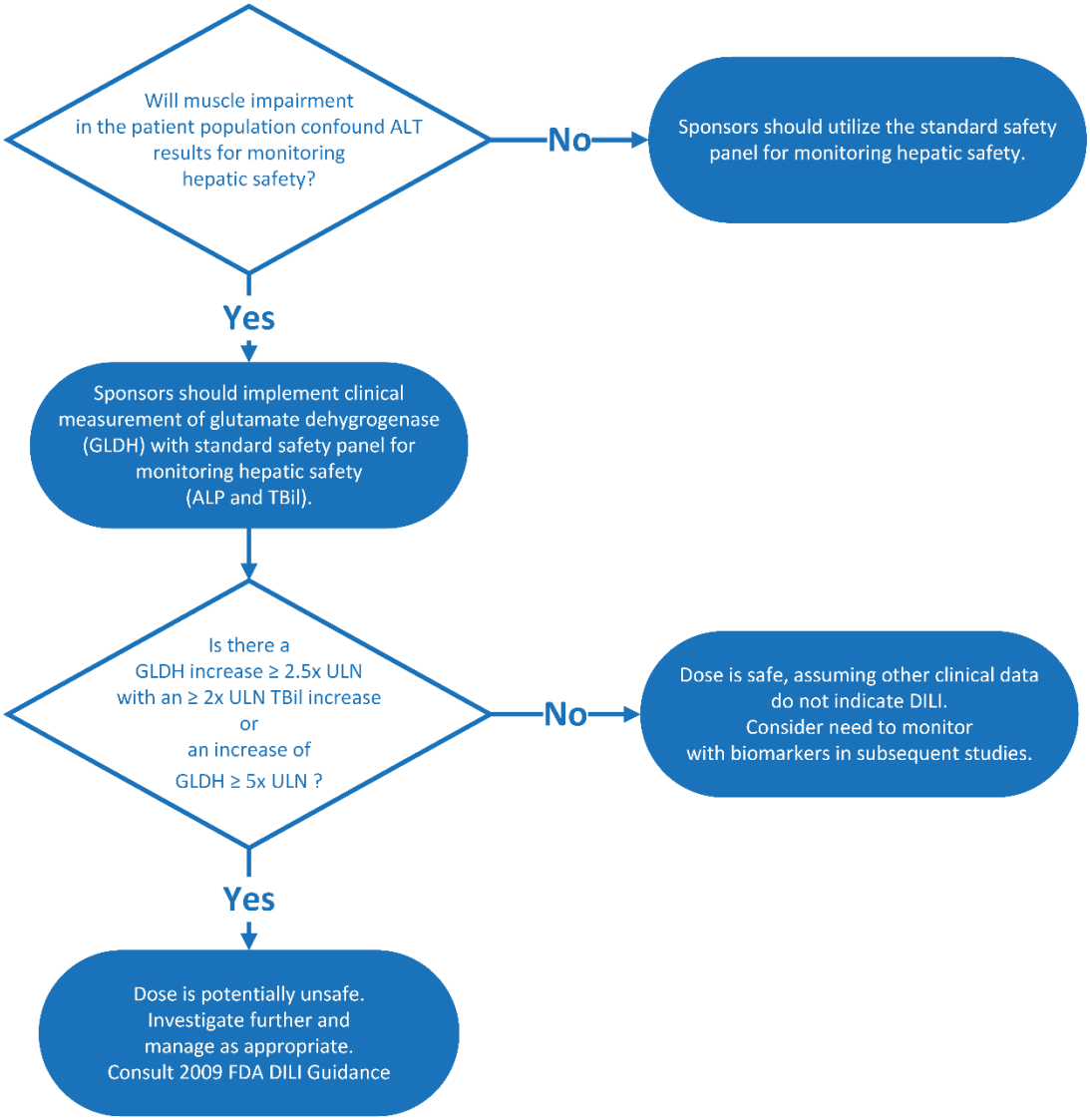
The Randox GLDH assay kit is manufactured in the United Kingdom (UK) with ISO13485 certification as evidence of Good Manufacturing Practice (GMP). The Randox GLDH assay was validated according to Centers for Medicare and Medicaid Services' (CMS) Clinical Laboratory Improvement Amendments (CLIA) guidelines for Laboratory Developed Tests (LDT). The assay is an approved *in vitro* diagnostics (IVD) assay in Europe, Health Canada, and China. The UK manufacturing facility was FDA inspected in 2012 and 2013.

1.6 Drug Development Clinical Interpretation/Decision Process Including Steps and Elements of Decision-Making Process and Cut-off Points/Limits/Thresholds

A 3x or 5x change from ULN for ALT is the currently accepted benchmark of concern for possible DILI in clinical trials. The mechanism by which GLDH and ALT increase in serum following

hepatocellular injury is similar, and their enzymatic activity is highly correlated in humans (Schomaker et al., 2013; Harrill et al., 2012; Antoine et al., 2013) and animals (O'Brien et al., 2000; O'Brien et al., 2002; Giffen et al., 2002; Jackson et al., 2008; Luo et al., 2014) with a diversity of drug-induced liver injuries and diseases. Based on the analysis of the datasets described within this submission, an increase in serum GLDH of 2.5x and 5x will lead to the same actions in management of DILI as a 3x and 5x increase of serum ALT (FDA 2009). The FDA DILI guidance document also includes directives around higher serum concentrations of ALT (10x, 15x, and 20x) which are concordant with specific derived increases of GLDH (11.5x, 19x, and 27.5x). Therefore, these increases in serum GLDH should lead to the same actions in management of DILI as the corresponding increases in serum ALT.

Decision Tree for Clinical Use of GLDH



1.7 Summary of Analytical Validation

1.7.1 Completed Analytical Validation Studies and Results

The Randox GLDH assay was validated according to CMS CLIA guidelines for LDT. As required by CLIA, the laboratory determined performance specifications and was responsible for the quality of the results generated from the test. During assay validation the following parameters were tested: accuracy, precision, analytical sensitivity (limit of blank (LOB)), long term stability, freeze/thaw stability, analytical specificity to include interfering substances, reportable range, and reference interval. The GLDH assay parameters are shown in the table below. Appropriate quality control samples were applied during the validation procedure and throughout the subsequent sample analysis to ensure data reliability and data comparability over the different clinical studies included in this package. Described below are the most current analytical validation parameters for the clinical GLDH assay.

Precision: The GLDH human serum assay demonstrated acceptable precision. Analyzed human serum samples and quality control material demonstrated appropriate assay precision (<10% coefficient of variation (CV)). However, at the low end of the linear range, there were several points that did not meet the predefined acceptance criteria. This resulted in a change in the validated dynamic range of the assay and increase in the lower limit of quantitation (LLOQ) from 1 to 3 U/L.

Linearity: The GLDH human serum assay demonstrated acceptable linearity. Samples had appropriate percent recovery values down to 3 U/L on the Siemens Advia 1800 Chemistry Analyzer. Assay linearity was established from 3 to 500 U/L on this instrument.

Recovery: The GLDH human serum assay demonstrated acceptable recovery. All samples had appropriate percent recovery values (80-120%) based upon the results of this testing and the acceptance criteria.

Reference Interval: The reference range interval for GLDH in human serum is < 3 to 10 U/L.

Sample Freeze/Thaw Stability: All samples had appropriate percent recovery (80-120% of initial thaw). Based upon the results of this testing and the acceptance criteria, GLDH in human serum demonstrates acceptable stability for 4 freeze thaw cycles.

Sample Stability: Most samples had appropriate percent recovery (80 to 120%). Based upon the results of this testing and the acceptance criteria, GLDH in human serum demonstrates acceptable stability at room temperature up to 48 hours, refrigerated up to 14 days, and frozen at -80°C up to 36 months.

Interference: All samples met the acceptance criteria except for lipemia. At high concentrations of triglycerides (>1288 ng/ml) there is interference with GLDH testing. Hemolysis (hemoglobin) and icterus (bilirubin) did not interfere with the measurement of GLDH.

Summary of GLDH Assay Performance Characteristics for Human Serum

Platform	Siemens Advia 1800 Chemistry Analyzer
Assay Vendor	Randox
Detection	Kinetic Enzymatic Assay (NADH, 340 nm)
Units	U/L[†]
	Mean ± Standard Deviation (%CV[†])
Precision – LOD[†]	2 ± 0.89 (37.7) U/L
Precision – Low	7 ± 0.89 (13.0) U/L
Precision – QC[†] Level 2	16 ± 1.36 (8.0) U/L
Precision – QC[†] Level 3	29 ± 1.36 (4.7) U/L
Precision – Mid	53 ± 2.43 (4.6) U/L
Precision – Conc. Calibrator 3	59 ± 3.27 (5.6) U/L
Precision – High	623 ± 15.37 (2.5) U/L
LLOQ[†]	3 U/L
ULOQ[†]	500 U/L
Upper reportable limit	125,000 U/L
Recovery range	85 –118%
Reference interval	<3 – 10 U/L
Dilutional range	1:250
Interferences – No Effect	Hemolysis (Hemoglobin) Icterus (Bilirubin)
Interferences – False Negative	Lipemia (Triglyceride; >1288 ng/ml)
Interferences – False Positive	None Determined

[†]CV: Coefficient of variation, LOD: Limit of detection, NADH: nicotinamide adenine dinucleotide (NAD) + hydrogen (H), QC: Quality control, LLOQ: Lower limit of quantitation, ULOQ: Upper limit of quantitation, U/L: units/liter

1.7.2 Planned Analytical Validation Studies and Methods

All analytical validation experimentation has been completed.

1.8 Summary of Clinical Considerations in Support of Biomarker's COU

1.8.1 Summary of Completed Studies/Analysis

1.8.1.1 Establish GLDH Reference Range

- **Objective:** Establish GLDH reference range for healthy subjects and evaluate influence of sex, ethnicity, and age.
- **Study design:** Serum samples were collected from either healthy volunteer subjects meeting recruitment criteria for Phase I trials, healthy subjects with normal liver function, or healthy volunteers meeting recruitment criteria for the PSTC biomarker study.
- **Statistical analysis:** To establish a reference range, a nonparametric approach was used, as recommended in Clinical and Laboratory Standards Institute (CLSI) document C28-A2 (CLSI, 2000). More specifically, the 2.5th and 97.5th percentiles were computed using a population of healthy subjects.

- **Results:** The GLDH reference range for this population (n = 665) was < 3 to 10 U/L and encompasses 97.5% of the population. The ULN of 10 U/L is used for further analyses.

1.8.1.2 Establish Sensitivity of GLDH and Correlation with ALT

- **Objective:** Establish GLDH as a sensitive marker of hepatotoxicity that strongly correlates with ALT.
- **Study design:** Serum samples were utilized from subjects with AST and ALT > 2x ULN and with diagnosed disease or injury resulting in increased liver enzymes (total n = 479). All healthy subjects used in the reference range study were included in the analysis.
- **Statistical analysis:** To assess the ability of GLDH to predict DILI, a receiver operating characteristic (ROC) curve was computed using a combined group (n = 758) of hepatic injury subjects and healthy subjects. Specifically, a logistic regression model was constructed using the presence of DILI as defined by the EWG as the response (141 subjects with DILI, 617 without DILI) and GLDH levels as the explanatory variable.
- **Results:** The Pearson correlation coefficient, computed using log[GLDH] and log[ALT], was $r^2 = 0.93$ (p-value < 0.001), indicating a strong positive correlation. The area under the ROC curve was 0.987.

1.8.1.3 Establish that GLDH in Not Affected by Muscle Injury in Rhabdomyolosis Patients

- **Objective:** Establish GLDH as biomarker that is not specific to muscle injury.
- **Study design:** Serum samples from (a) healthy subjects (n = 125; 3-64 years of age) and (b) subjects with muscle injury (n = 131; 2-78 years of age) were utilized to examine the specificity of GLDH for liver injury in human subjects with muscle impairment and determine if GLDH can detect liver injury onset in rhabdomyolysis patients (case study).
- **Statistical analysis:** To evaluate the lack of muscle specificity of serum GLDH, the correlation of serum ALT and GLDH with serum creatine kinase (CK), a widely used biomarker of muscle injury, was examined.
- **Results:** GLDH and CK had a poor correlation value of $r^2 = 0.20$ suggesting a lack of GLDH muscle specificity. There was a good correlation ($r^2 = 0.68$) between ALT and CK.

1.8.1.4 Establish that GLDH is Not Affected by Muscle Injury in Duchenne muscular dystrophy (DMD) Patients

- **Objective:** Establish GLDH as biomarker that is not specific to muscle injury.
- **Study design:** Serum samples were collected from DMD patients (n = 40; 5-14 years of age) to evaluate the liver specificity of serum GLDH in subjects with muscle impairment and to characterize GLDH levels in DMD patients.

- **Statistical analysis:** Serum ALT, AST, CK and serum GLDH activity levels in DMD boys were compared to serum ALT, AST, CK and GLDH activity levels in healthy boys from the reference range dataset to evaluate the lack of muscle specificity of serum GLDH.
- **Results**: Elevations in serum activities of ALT (~19x), AST (~11x), as well as CK (~107x), a commonly used biomarker of muscle damage, were observed in DMD boys. The serum GLDH activity was within the reference range in DMD boys (Mean = 5, standard deviation (SD) = 2) and was not different when compared to healthy boys (Mean = 4, SD = 2).

1.8.1.5 Establish that GLDH is Not Affected by Extreme Exercise

- **Objective:** Determine whether GLDH changes with exercise.
- **Study design:** Serum samples were taken pre- and post-race from subjects (n = 12) participating in an extreme adventure race.
- **Statistical analysis:** To determine whether there was a statistically significant difference between pre- and post-race samples, a Wilcoxon matched pairs test was used to compare ALT, AST, CK and GLDH levels.
- **Results:** ALT, AST, and CK levels were substantially elevated in the serum of the participants following an extreme adventure race, whereas GLDH remained unchanged.

1.8.1.6 Nonclinical Support for Establishing Sensitivity and Specificity of GLDH

- **Objective:** Establish GLDH as biomarker specific to liver injury in the rat using histopathological evidence of hepatocellular injury.
- **Study design:** Histopathology and biomarker data (ALT, AST, and GLDH) were collected from rats treated with well characterized toxicants (30 individual toxicants), including those targeting liver, kidney, heart, and pancreas. Furthermore, histopathology and biomarker data (ALT, AST, and GLDH) were collected from rats treated with acetaminophen (APAP) and rats treated 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD), a known muscle toxicant.
- **Statistical analysis:** To enable an estimate of sensitivity and specificity of GLDH to detect hepatocellular necrosis using histopathology a ROC curve was constructed.
- **Results:** GLDH is a sensitive and specific biomarker of hepatocellular liver injury as determined by histopathology. In this set of animal studies, GLDH performed similarly to ALT for the detection of hepatocellular necrosis (area under the ROC curve for GLDH = 0.844; ALT = 0.823).

1.8.2 Proposed Studies/Data Analysis Plan for Biomarker Qualification

1.8.2.1 Confirmation of the Linear Relationship of ALT and GLDH in Humans

- **Objective:** Confirm the performance of GLDH as a biomarker of liver injury, as well as confirm the performance of GLDH thresholds of concern to identify subjects with liver injury.
- **Study design:** Serum levels of GLDH and ALT will be evaluated in healthy subjects and subjects with liver injury featuring a wide variety of etiologies. The study population will comprise approximately 200 samples from heathy subjects and 200 samples from subjects with liver injury, including subjects with acetaminophen overdose and two additional investigational drugs that cause liver injury. The ability of GLDH to substitute for ALT in the determination of liver injury will be confirmed across the entire data set.
- **Statistical analysis:** 2x2 contingency tables of the EWG definition of liver injury using ALT will be compared to replacing ALT with GLDH. Measures of concordance, sensitivity, and specificity of the GLDH-based EWG definition of liver injury will be then calculated. These measures will be used to predict the ALT-based EWG definition of liver injury using proposed GLDH thresholds and computed ALT thresholds determined in the exploratory studies. The target success for each measure is ≥ 0.90, 95% Lower Confidence Bound ≥ 0.85.

1.8.2.2 Confirmation that GLDH Does Not Increase with Muscle Injury in Humans

- **Objective:** Confirm that serum GLDH levels are unaffected by muscle injury in humans, and that GLDH outperforms ALT with regard to specificity for liver injury.
- **Study design:** Serum levels of GLDH and ALT will be compared in healthy subjects and subjects with muscle impairments featuring a wide variety of etiologies. Serum samples from approximately 120 subjects with adjudicated muscle injury will be selected. Muscle injury will be defined by CK levels greater than two times normal healthy levels or based on a diagnosed muscle injury.
- **Statistical analysis:** The primary endpoint is the false positive rate (FPR), i.e., the % of subjects exceeding a prespecified threshold defined below. The percentage of subjects with GLDH values exceeding ULN (10 U/L), 2.5x ULN (25 U/L) and 5x ULN (50 U/L) will be computed. The target success criteria are:
    - ULN: FPR $\leq$ 10%
    - 2.5x ULN: FPR $\leq$ 5%
    - 5x ULN: FPR $\leq$ 1%

1.8.2.3 Confirmation of the Specificity of GLDH for Liver Injury in Humans with Pancreatic, Gastrointestinal, or Kidney Injury

- **Objective:** Confirm that GLDH does not increase with pancreatic, gastrointestinal, or kidney injury.
- **Study design:** Serum GLDH levels from subjects with pancreatic, gastrointestinal, or kidney injury will be compared to healthy volunteers. Approximately 200 serum samples from subjects with pancreatic, gastrointestinal, and kidney injury will be collected for this study. The serum concentration of GLDH from subjects with pancreatic, gastrointestinal, or kidney injury will be compared to healthy volunteer samples to confirm that these injuries do not affect serum GLDH levels, and thereby do not interfere with the ability of GLDH to detect liver injury.
- **Statistical analysis:** The primary endpoint is the FPR. Specifically, the percentage of subjects with GLDH values exceeding 2.5x ULN (25 U/L) and 5x ULN (50 U/L) will be computed. The target success criteria are:
    - 2.5x ULN: FPR $\leq$ 5%
    - 5.x ULN: FPR $\leq$ 1%

1.8.2.4 Further Characterization of the Elimination Kinetics of GLDH and ALT in Humans

- **Objective:** Confirm that GLDH detects liver injury caused by acetaminophen (APAP) overdose and that the half-life of GLDH, which is shorter than the half-life of ALT in human serum, reflects the time course of hepatocellular injury.
- **Study design:** The time course of GLDH activity in human serum will be compared with ALT activity and the medically adjudicated clinical outcome of APAP poisoning. Approximately 15 cases of APAP overdose will be evaluated.
- **Statistical analysis:** A visual comparison of each subject's serum GLDH level to the ULN will be made at hospital admission. Target success criteria will be that all subjects have GLDH greater than ULN.

1.8.2.5 Additional Nonclinical Support for the Use of GLDH to Detect Liver Injury During Concurrent Muscle Injury

- **Objective:** Further assess the specificity of serum GLDH for detection of liver injury by evaluating the performance of GLDH following drug-induced hepatocellular injury in a genetic mouse model of muscular dystrophy (MD).
- **Study design:** This genetic mouse model of increased ALT due to muscle injury/disease, mimics the proposed COU for GLDH use. Acetaminophen will be used to induce liver injury. Liver and muscle histopathology and serum chemistry analysis including ALT, AST, CK and GLDH will be completed to demonstrate that, in contrast to serum ALT,

serum GLDH is a liver-specific biomarker of liver injury and is not affected by muscle damage.

- **Statistical analysis:** The time course of the response of the biomarkers will be evaluated and compared to histopathology.