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Research article

Serum glutamate dehydrogenase activity enables sensitive and specific diagnosis of hepatocellular injury in humans

Jiri Aubrecht [1], David Potter[2], John Michael Sauer [3], Roscoe Warner[4], Kent J. Johnson[4], Mitchell R. McGill [5], Katrina Peron [3,*], Nicholas M.P. King [3]

[1]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20007, United States
[2]Nonclinical Statistics, Pfizer R&D, Cambridge, MA 02139, United States
[3]Predictive Safety Testing Consortium, PSTC, Critical Path Institute, Tucson, AZ 85718, United States
[4]Department of Pathology, University of Michigan, Ann Arbor, MI 48109, United States
[5]Department of Environmental Health Sciences, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

*Corresponding author: Predictive Safety Testing Consortium (PSTC), Critical Path Institute, 1840 E River Rd, Suite 100, Tucson, AZ 85718, United States. Email: kperon@c-path.org

Abstract

Serum activities of alanine- and aspartate aminotransferases (ALT and AST) are considered the "gold standard" biomarkers of hepatocyte injury in clinical practice and drug development. However, due to the expression of ALT and AST in myocytes, the diagnosis of hepatocellular injury in patients with underlying muscle diseases, including drug-induced muscle injury, is severely limited. Thus, we proposed glutamate dehydrogenase (GLDH) as a liver-specific alternative to serum ALT and AST. In fact, our exploratory studies showed that GLDH has comparable performance to ALT for detecting hepatocyte injury without interference from concomitant muscle injury. Here, we report the results of studies confirming the reference intervals in a healthy human population and the sensitivity and specificity of GLDH for the detection of hepatocyte injury in human subjects. In human subjects, we could not perform liver biopsies due to ethical reasons; we also confirmed the relationship of GLDH and histopathologic lesions using 32 model toxicants in rats. Furthermore, we have shown that injury to tissues that are known to express appreciable levels of GLDH does not affect serum GLDH measurements, indicating excellent liver specificity of serum GLDH. Finally, we observed faster elimination of GLDH than ALT in humans, indicating that decreasing GLDH values could be considered an early sign of recovery. This study provides comprehensive evidence of excellent sensitivity and liver specificity of GLDH for diagnosis of hepatocellular injury, including evaluation of reference intervals, which is essential for the interpretation of serum GLDH in human subjects.

Keywords: hepatotoxicity; liver; organ toxicity; predictive toxicology; translational; safety evaluation

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum activities are considered the gold standard biomarkers for diagnosis of hepatocellular injury in both clinical practice and drug development (Amacher et al. 2013). As a consequence of compromised cell membrane integrity upon hepatocyte injury, ALT and AST leak into systemic circulation where they are easily detected by their enzymatic activities (Karmen et al. 1955). Due to the large functional reserve capacity of the liver, transaminase increases precede the functional changes that manifest as increased serum bilirubin and coagulation time. Thus, transaminases are especially important for the early diagnosis of hepatocellular injury before it has progressed to liver failure.

Although ALT and AST have proven to be sensitive markers of hepatocellular injury, both enzymes lack liver specificity. Large amounts of these enzymes are present in other tissues, with the highest extrahepatic expression in myocytes. Clinically, this severely limits the utility of ALT and AST as markers of hepatocellular injury in subjects with underlying chronic or acute muscle impairments, such as Duchenne muscular dystrophy, myositis, rhabdomyolysis, and drug-induced muscle injury (Schomaker et al. 2020). Persistent transaminasemia is frequently misdiagnosed as hepatocellular injury in patients with such conditions, leading to additional unnecessary tests and causing delays in diagnosis and treatment of the neuromuscular disease (Rutledge et al. 1985; Begum et al. 2000; Nathwani et al. 2005). Similarly, the lack of liver specificity of ALT severely limits hepatocellular injury detection in subjects with underlying muscle impairments in clinical trials for new therapies to treat muscle disorders. Additionally, several widely used therapeutics, such as statins and fibrates, are known to cause muscle toxicity. When these drugs are taken concomitantly during clinical trials, the interpretation of increases in serum ALT, particularly those that are relatively small and transient, can be challenging. This can be complicated even further because significant increases in ALT and AST can occur even in healthy subjects after strenuous

exercise and changes in diet (Apple and Rogers 1984; Purkins et al. 2004; Pettersson et al. 2008; Wei et al. 2019). Therefore, the development of liver-specific biomarkers of hepatocyte injury is an important unmet medical need.

Here, we propose the use of glutamate dehydrogenase (GLDH) as a liver-specific biomarker to rule out hepatocellular injury when ALT is elevated in the setting of neuromuscular disease or muscle damage. GLDH is a mitochondrial enzyme that plays a role in amino acid oxidation and urea production. It is primarily found in the liver, with only a trace amount in skeletal muscle (Mastorodemos et al. 2005). We and others have demonstrated that serum GLDH is a sensitive biomarker of hepatocyte injury in patients with a variety of liver diseases (Schomaker et al. 2013), including hepatic ischemia (Schmidt and Schmidt 1988; Kretzschmar et al. 2003), APAP overdose (McGill et al. 2012; Schomaker et al. 2013; Antoine et al. 2014), mild hepatocyte necrosis after treatment with heparin (Harrill et al. 2012), and alcohol-induced injury (Kravos and Malešič 2008; Kravos and Malesic 2010). Importantly, however, GLDH did not increase following muscle injury or degeneration or in response to strenuous exercise (Thulin et al. 2014; Schomaker et al. 2020). These studies served as the foundation for initiating a formal biomarker qualification at the FDA and EMA (https://www.fda.gov/drugs/biomarker-qualification-program/biomarker-qualification-submissions).

In the present study, we set out to build on our prior results by establishing reference intervals in a healthy human population, and to determine the sensitivity and specificity of GLDH for hepatocyte injury in human subjects with a variety of liver diseases. Since, in human subjects, we could not perform liver biopsies due to ethical reasons, we also confirmed the relationship between GLDH and hepatic histopathologic lesions in a series of studies in rats. To complete the assessment of liver specificity of serum GLDH, we then measured serum GLDH levels in patients with GI, pancreas, or kidney impairments, all known organs with appreciable GLDH expression. Finally, to facilitate interpretation of GLDH values, i.e. time course, we have assessed the elimination kinetics of GLDH. Overall, our study provides comprehensive evidence for the excellent sensitivity and liver specificity of GLDH for the diagnosis of hepatocellular injury in humans. We also provide general guidance for the interpretation of serum GLDH data (Aithal et al. 2011; Schomaker et al. 2013, 2020).

Materials and methods

Acquisition of samples from healthy subjects

Blood samples from 125 healthy subjects were collected from the University of Michigan health care system (UM) under an approved IRB (HUM0044422). Researchers did not have access to any potentially identifying personal information. The University of Michigan IRB committee waived the requirement for informed consent for this sample-set collection, for current and future unspecified project usage. Samples were defined as healthy based on normal levels of ALT, AST, alkaline phosphatase (ALP), total bilirubin (TBil), glucose, blood urea nitrogen, serum creatinine, and creatinine kinase (CK). Subjects whose values for one or more of the above endpoints exceeded the normal reference range were not used in this study. In addition, any healthy subject that had an ongoing health problem or immunological flare was omitted from the cohort. Most samples were collected from subjects who were at the University of Michigan for routine health examinations.

All samples in study HUM0044422 were collected between July 12, 2013 and April 2, 2019.

Acquisition of samples from subjects with liver, muscle, kidney, pancreas, or GI injury

Blood samples from subjects with liver, muscle, kidney, pancreas, or GI injury were collected from the University of Michigan health care system (UM) under an approved IRB (HUM0044422). Researchers did not have access to any potentially identifying personal information. Dr K. Johnson and Dr R. Warner provided medical adjudication of subjects from UM and had access to medical records as required by UM. The University of Michigan IRB committee waived the requirement for informed consent for this sample-set collection, for current and future unspecified project usage. Samples for hepatocellular injury featured abnormal hepatic enzyme profiles such as AST and ALT levels greater than 2 times normal healthy levels and with a diagnosed disease resulting in impaired liver function were grouped and loosely categorized as liver transplant (liver transplant within the last 3 yr), hepatic carcinoma (diagnosed by biopsy or resection), coronary artery disease–coronary heart disease (aortic aneurism, myocarditis, atrial mass, aortic valve replacement, or heart catheterization), cirrhosis and hepatocellular injury (Hepatitis B or C, hepatic graft vs host disease, ethanol cirrhosis, drug abuse, or transaminitis/hepatic congestion), pulmonary (Influenza A, H1N1 Influenza, acute respiratory distress syndrome, or latent tuberculosis), and acetaminophen toxicity (APAP-induced liver failure). Samples classified as muscle injury featured abnormal CK enzyme activity levels or clinically demonstrable muscle injury as assessed by medical adjudication. Clinically determined injuries could include, but were not limited to, primary disorders of muscle (dystrophies, myotonic disorders, congenital myopathies, and mitochondrial myopathies) and toxic myopathies (drug, alcohol, and toxicants), as exhibited by myositis (inflammatory muscle injury), neurogenic atrophy, necrotizing inflammatory muscle injury, chronic severe atrophy, angulated atrophic fibers, type II-fiber atrophy, nuclear myobags, denervation atrophy, and/or increased lipids in myofibers. Additional comorbidities were often present. Samples from patients with hepatocellular injury were excluded from this analysis because GLDH would be expected to be elevated in these subjects due to hepatocellular injury. For the specificity analysis (muscle, kidney, pancreas, or GI injury studies), subjects with hepatocellular injury based on the clinical chemistry criteria (>5× ALT or >2× ALP or [>3× ALT and >2× Tbil]) or evidence of hepatocellular injury in the medical records were excluded.

Patients classified as having kidney injury were medically adjudicated as having chronic kidney disease (CKD) diagnosed by either (i) Biopsy-Proven or (ii) Clinically Demonstrable Deficiencies, which could include, but are not limited to, Diabetes, High Blood Pressure, Glomerulonephritis, Interstitial Nephritis, Polycystic Kidney Disease, and Malformations, as exhibited by CKD stage II–V, end-stage renal disease and patients on dialysis.

Patients with pancreatic injury were medically adjudicated as having pancreatitis (Acute, Chronic, Hereditary) that is diagnosed by either (i) Persistent Severe Epigastric Pain, (ii) Diagnostic Armamentarium (Endoscopic Ultrasound, Magnetic Resonance Cholangiopancreatography, Computerized Tomography, or Transabdominal ultrasound), (iii) Clinically Demonstrable Deficiencies, or (iv) Amylase or Lipase 3X upper limit of normal (ULN).

Patients with gastrointestinal injuries were medically adjudicated as having gastrointestinal abnormalities diagnosed by

either (i) Endoscopy, (ii) Sigmoidoscopy or (iii) Colonoscopy, or (iv) Clinically Demonstrable Deficiencies, which could include, but are not limited to, Gastroesophageal Reflux Disease, Esophagitis, Irritable Bowel Syndrome, Celiac Disease, Crohn's Disease, Ulcerative Colitis, Ulcerative Pancolitis, Ulcerative Proctosigmoiditis, and Appendicitis.

Additional samples were collected from acetaminophen (APAP) overdose patients at the University of Kansas Medical Center (IRB 11962). All patients or next of kin were informed of the study objectives and design by a physician and signed a consent form. All patients were required to meet at least 2 of the following inclusion criteria: (i) a reported history of APAP overdose, (ii) high plasma APAP levels based on the Rumack–Matthew nomogram, and (iii) both ALT >1,000 U/l and prothrombin time ≥18 s. Patients were excluded if there was reasonable evidence of hepatocellular injury due to another etiology (e.g. acute viral hepatitis) or if they were <18 yr of age.

All samples in study HUM0044422 were collected between July 12, 2013 and April 2, 2019. All samples collected at the University of Kansas Medical Center (IRB 11962) were originally published in McGill et al. (2012). A subset of samples reported here was published in the Llewellyn et al. paper. The breakdown of each injury type subset used in the Llewellyn et al. paper out of the total confirmatory subjects included here is as follows: 135/294 healthy subjects, 104/197 liver disease, 74/128 muscle injury, 34/57 pancreatic injury, 37/118 gastrointestinal injury, and 40/62 kidney injury.

Sample preparation and analysis

Serum samples were recovered from serum-separator tubes following centrifugation of whole blood at 3000 × *g* for 10 min at room temperature. Serum samples were kept at 4 °C for up to 72 h before aliquots were frozen at −80 °C and stored until shipped for biomarker analysis. A stability assessment of GLDH, MDH, PNP, and PON1 confirmed acceptable stability at 4 °C for up to 96 h. All analytes were measured in serum on the Siemens ADVIA 1800 or ADVIA 2400 Automated Chemistry System at Pfizer, Groton, Connecticut. ALT and AST (Siemens) and GLDH (Randox Labs Ltd, Roche) were measured using commercially available kits within the stability range (within 18 mo per Table S1). MDH, PNP, and PON1 were determined according to Bergmeyer *et al.* (1983), Chu *et al.* (1989), and Li *et al.* (1995), respectively. The GLDH assay completed validation in a CLIA-certified lab. A summary of the GLDH assay validation results, including validation acceptance criteria and assay performance characteristics, is included in Tables S1 and S2, respectively.

Statistical analysis

Reference ranges were computed using the nonparametric approach recommended in the Clinical Laboratory Standards Institute (CLSI) document C28-A2 (CLSI 2000). ULNs were compared across subgroups using quantile regression (Koenker 2005). Linear regression was used to assess the correlation between ALT and GLDH, and to estimate GLDH thresholds of concern corresponding to those used with ALT in the Expert Working Group (EWG) definition of hepatocellular injury. Logistic regression and ROC curve analysis were used to assess the predictivity of GLDH as a predictor of hepatocellular injury. Statistical analyses were conducted in R (R Core Team 2021).

Rat studies

Data were obtained from a variety of PSTC member companies conducting toxicology studies investigating liver biomarkers in rodents (Bailey et al. 2012). Generally, studies utilized multiple doses to achieve a diversity of injury diagnoses and severity; all studies utilized a vehicle-treated control group. All sites used a common lexicon developed by pathologists within the PSTC Hepatotoxicity Working Group (HWG) to define histopathology diagnoses and severity. A histopathology diagnosis of hepatocellular necrosis, graded 0 to 4 in severity, was used for the purposes of detection of hepatocellular-based DILI. Blood samples were obtained prior to necropsy. Serum samples were recovered according to the standard operating procedures at the member company conducting the study.

The table in Supplementary Material S1 lists the model toxicants evaluated in the rat to define the sensitivity and specificity of GLDH as a biomarker to detect hepatocellular injury. As with nearly all toxicants, target organ/tissue toxicity is dose and route of administration dependent. Therefore, we have listed the toxicants based on their primary target organ/tissue. Please note that histopathological evaluation was used to define whether or not a toxicity occurred in an individual animal.

Linear regression was used to assess the correlation between ALT and GLDH. Logistic regression and ROC curve analysis were used to assess the predictivity of GLDH and ALT as predictors of hepatocellular necrosis.

Results

Establishing the reference interval for serum GLDH in human subjects

A detailed understanding of reference intervals in healthy human subjects, including the effects of gender, age, and ethnicity, is essential for the interpretation of biomarker data. To characterize reference intervals in healthy human subjects, we calculated the mean, median, and standard deviation of GLDH, as well as a 97.5% reference interval using the nonparametric approach recommended in the Clinical Laboratory Standards Institute (CLSI) document C28-A2 (CLSI 2000). Then, we compared the reference interval parameters across 2 datasets, one from an initial exploratory population and one confirmatory, and evaluated the effects of age, gender, and ethnicity. To facilitate the acceptance of GLDH as a liver-specific alternative to ALT, we also compared GLDH reference intervals with those for the gold standard biomarker ALT. Our exploratory population included 665 healthy subjects combined from 3 separate cohorts from prior studies (Schomaker et al. 2013, 2020) and the new confirmatory study (presented here) included data from 294 healthy subjects (Table 1). Although we have previously published some data from the exploratory cohorts, all data and analyses presented in this manuscript are new.

The mean and median serum activities of serum GLDH were almost identical across all cohorts in both the exploratory and confirmatory studies. The ULN in the exploratory population was 10 U/l and this cutoff was supported by the confirmatory group, which had a similar ULN of 7 U/l. Combining all groups resulted in an overall ULN of 9 U/l. Because the exploratory population was larger and more diverse than the confirmatory population, we chose 10 U/l as our ULN moving forward. Median, mean, and ULN values for ALT proportionally mirrored the GLDH levels across the datasets, with the lowest ULN in the confirmatory study (28 U/l) and higher in the exploratory study (41 U/l). Combining both datasets resulted in an overall ULN of 39 U/l. Statistical tests comparing the ULN values across studies revealed a statistically significant difference in ALT ($P < 0.001$) but not GLDH ($P = 0.14$). Even for ALT, however, the differences

Table 1. GLDH and ALT values in healthy subjects across studies and demographic parameters.

		GLDH				ALT			
Subgroup	**N**	**Mean**	**SD**	**Median**	**97.5th%**	**Mean**	**SD**	**Median**	**97.5th%**
All subjects	959	3.3	2.2	3	9.0	20.3	7.6	19	39.0
Confirmatory study	294	3.5	1.2	3	7.0	16.1	5.1	16	28.0
Exploratory study	665	3.3	2.5	3	10	22.1	7.8	21	41
Age <20 yr	98	3.3	1.3	3	6.0	16.3	6.0	14	33.2
Age 20 to 40 yr	373	3.2	2.1	3	8.0	19.7	7.6	18	40.0
Age 41 to 60 yr	366	3.6	2.7	3	11.0	21.7	8.0	20	40.9
Age >61 yr	122	3.0	1.7	3	6.0	21.0	6.3	20	34.0
Ethnic origin									
AfrAmer	153	3.6	2.6	3	10.0	22.3	9.6	20	44.4
Asian	90	3.6	1.4	3	7.8	15.2	5.0	14	26.8
Caucasian	609	3.2	2.1	3	8.0	20.2	6.9	19	35.0
Hispanic	35	3.6	3.4	3	9.8	23.9	10.3	21	45.6
Other/Unknown	72	3.5	2.4	3	11.5	20.6	6.5	21	35.7
Sex									
F	490	3.1	2.0	3	8.0	18.2	6.3	17	33.0
M	469	3.5	2.4	3	9.0	22.4	8.3	21	42.3

across studies were not considered clinically significant to influence the interpretation of hepatocellular injury.

Effect of sex, age, and ethnicity on serum GLDH

Because sex, age, or ethnicity might affect biomarker values in healthy populations, we next evaluated reference range parameters across these categories in the combined (i.e. exploratory and confirmatory) dataset of 959 subjects (Table 1). The analysis of this dataset consisting of 490 female and 469 male subjects did not show any appreciable influence of gender on serum GLDH (ULN $P = 0.73$). In contrast, serum ALT mean, median, and ULN values were lower in females than males (33 U/l vs 42 U/l; ULN $P < 0.001$). The analysis of the effect of age on GLDH and ALT levels revealed that, for both serum GLDH and serum ALT, the ULN values trended lower in younger (<20 yo) and older (>61 yo) subjects (ALT ULN $P = 0.01$; GLDH ULN $P = 0.03$). Across ethnic groups, GLDH and ALT appeared to have similar patterns, including, for example, somewhat lower ULN values in the Asian subgroup. However, unlike ALT, the differences across ethnic subgroups were not statistically significant for GLDH ($P = 0.47$).

Intraindividual variability of serum GLDH

Our datasets included 2 to 4 repeat measurements of serum GLDH and serum ALT each over the course of 3 wk for 81 subjects and totaled 257 individual measurements. Figure 1 shows the repeat GLDH and ALT measurements relative to each subject's mean GLDH and ALT values, respectively. Within-subject variability, as measured by the standard deviation of repeat measurements, was 1.6 for GLDH, and 3.8 for ALT. Serum GLDH and serum ALT values from most subjects minimally changed over the 3-wk period. Of a total of 257 GLDH measurements, only 7 (2.7%) fell outside the reference range described above. In the case of ALT, 13 measurements out of 257 were above the ULN.

Sensitivity and specificity of serum GLDH for diagnosis of hepatocellular injury

To evaluate the diagnostic performance of GLDH for hepatocellular injury, we measured serum levels in a total of 338 patients (141 from the exploratory study; 197 from the confirmatory study) with chronic and acute liver diseases. Since liver biopsies were not ethically feasible, we utilized a biochemical criterion developed by the International DILI EWG (Aithal et al. 2011) to adjudicate hepatocellular injury. According to this biochemical criterion, hepatocellular injury is defined as any of the following 3 criteria: (i) an ALT value >3×ULN and TBil >2×ULN, (ii) ALT >5×ULN, or (iii) ALP >2×ULN. In a combined dataset of subjects with hepatocellular injury and healthy volunteers in our exploratory population, we observed a strong correlation between GLDH and ALT, shown in Fig. 2a (Pearson $R = 0.93$) (Schomaker et al. 2013). In the confirmatory study presented here, the correlation was 0.86. Combining the exploratory and confirmatory studies resulted in an overall correlation of 0.89.

To assess the sensitivity and specificity of serum GLDH alone to diagnose hepatocellular injury, we computed ROC curves using the exploratory studies (Schomaker et al. 2013). Specifically, a logistic regression model was constructed using the presence of DILI as defined by the EWG as the response (141 subjects with hepatocellular injury, 617 without hepatocellular injury) and GLDH levels as the explanatory variable. The resulting model was used to estimate sensitivity and specificity across the range of GLDH thresholds, yielding the ROC curve shown in Fig. 2b. The area under the ROC curve is 0.987, suggesting that GLDH has excellent discriminatory power to identify liver damage. In the confirmatory study presented here, the area under the ROC curve was 0.928 (not shown). Combining the exploratory and confirmatory studies resulted in an area under the ROC curve of 0.958 (not shown).

To estimate the serum GLDH diagnostic thresholds for hepatocellular injury, which correspond to commonly accepted serum ALT thresholds (3× and 5× ULN), we fit a simple linear regression model with log[GLDH] and log[ALT]. Based on data from the exploratory population, we used 10 U/l and 41 U/l as the ULN (i.e. 97.5th percentile) for serum GLDH and or ALT, respectively. As shown in Fig. 2a, the linear regression model identified 25 U/l and 48 U/l (2.5 and ~5× ULN) as serum GLDH thresholds of concern that corresponded to 3× ULN (123 U/l) and 5× ULN (205 U/l) thresholds of concerns for serum ALT. Confidence intervals (95%) for these cut-offs are (2.1, 3.4) and (3.9, 6.6), respectively.

Serum GLDH correlates with liver lesions

The rigorous assessment of serum GLDH performance to diagnose hepatocellular injury also requires histopathologic evaluation of liver tissues. Because obtaining liver biopsies from human subjects was not feasible due to ethical considerations, we evaluated the correlation of serum GLDH with liver lesions in rats. First, we evaluated the correlation of serum GLDH and serum

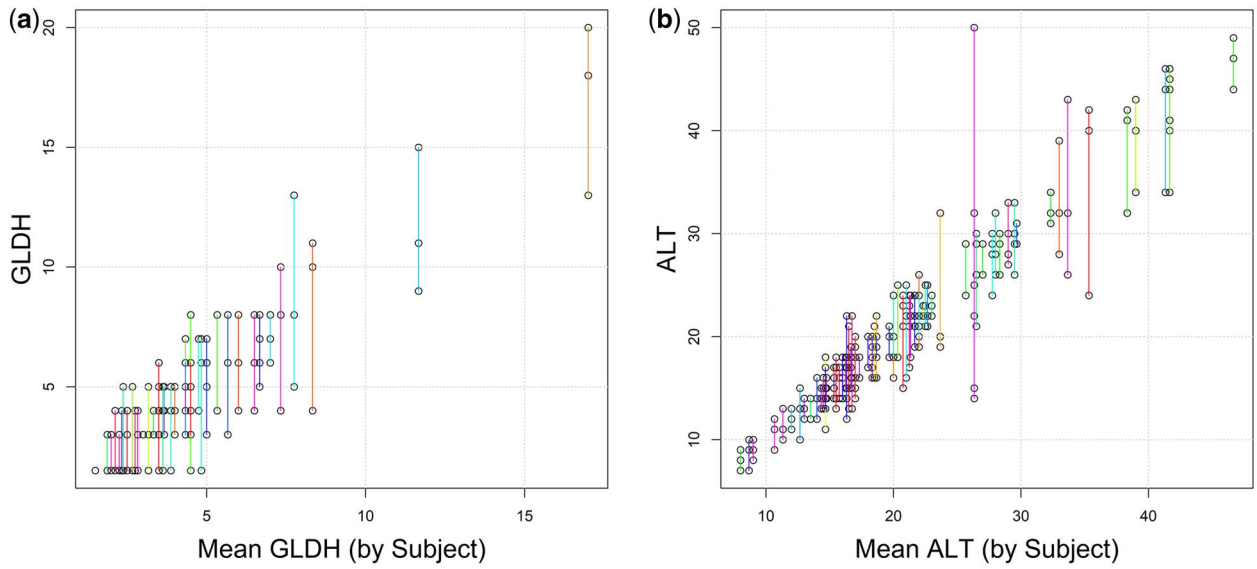

Fig. 1. Intra-individual variability of serum GLDH (a) and serum ALT (b). The bars with open circles represent average, minimum, and maximum values for 2 to 4 repeat measurements of serum GLDH (a) and serum ALT (b) for each of 81 subjects taken over the course of 3 wk. The dataset included 257 individual measurements. a) GLDH versus Mean GLDH (by Subject). b) ALT versus Mean ALT (by Subject).

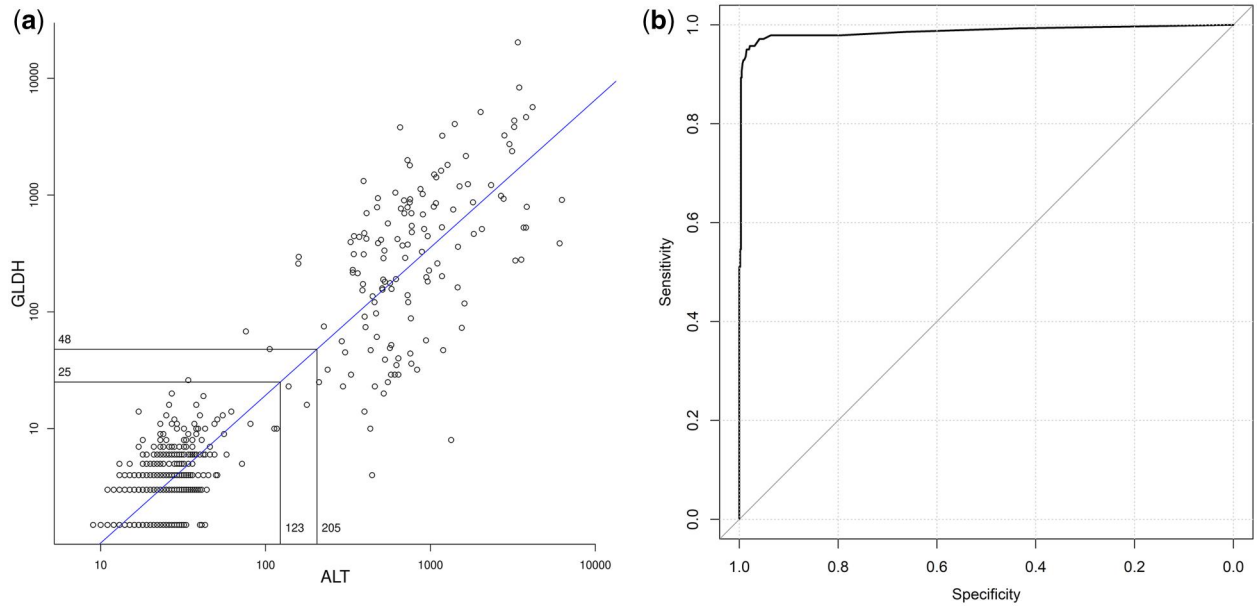

Fig. 2. Sensitivity and specificity of serum GLDH for diagnosis of hepatocellular injury. a) Data represent individual GLDH and ALT serum levels for a total of 338 patients with chronic and acute liver diseases and 294 healthy subjects. b) Assessment of serum GLDH to detect liver injury was performed using ROC analysis. Area under ROC = 0.987.

ALT and then compared their sensitivity and specificity to detect hepatocellular injury (Supplementary Material S1). To visualize the relationship between serum GLDH and serum ALT in the rats, we plotted their values on a log scale for animals that had normal histopathology ($n = 676$), and those that were positive for hepatocellular necrosis ($n = 222$), regardless of treatment group. However, from this group, we removed animals that were positive for skeletal muscle injury ($n = 5$) or had been treated with skeletal muscle toxicants ($n = 16$). The correlation of ALT and GLDH values from 877 rats is shown in Fig. 3.

As in human subjects, we observed a strong correlation between serum GLDH and serum ALT in rat studies ($r = 0.85$). To assess the sensitivity and specificity of serum GLDH to diagnose hepatocellular necrosis detected by histopathology, we computed ROC curves using the classification of hepatic necrosis regardless of its severity score. The precise estimation of the ROC curve was facilitated by a wide range of serum GLDH and serum ALT values across the treated and vehicle animals. As expected, the ROC analysis revealed excellent specificity and sensitivity of serum GLDH to detect hepatocellular necrosis caused by hepatotoxicants, which was almost identical to serum ALT. The areas under the curve (AUC) for GLDH and ALT were 0.84 or 0.83, respectively. The GLDH was not affected by muscle toxicants.

Assessment of liver specificity of serum GLDH in human subjects

In contrast to serum ALT, the serum GLDH is particularly useful for the diagnosis of hepatocellular injury in patients with

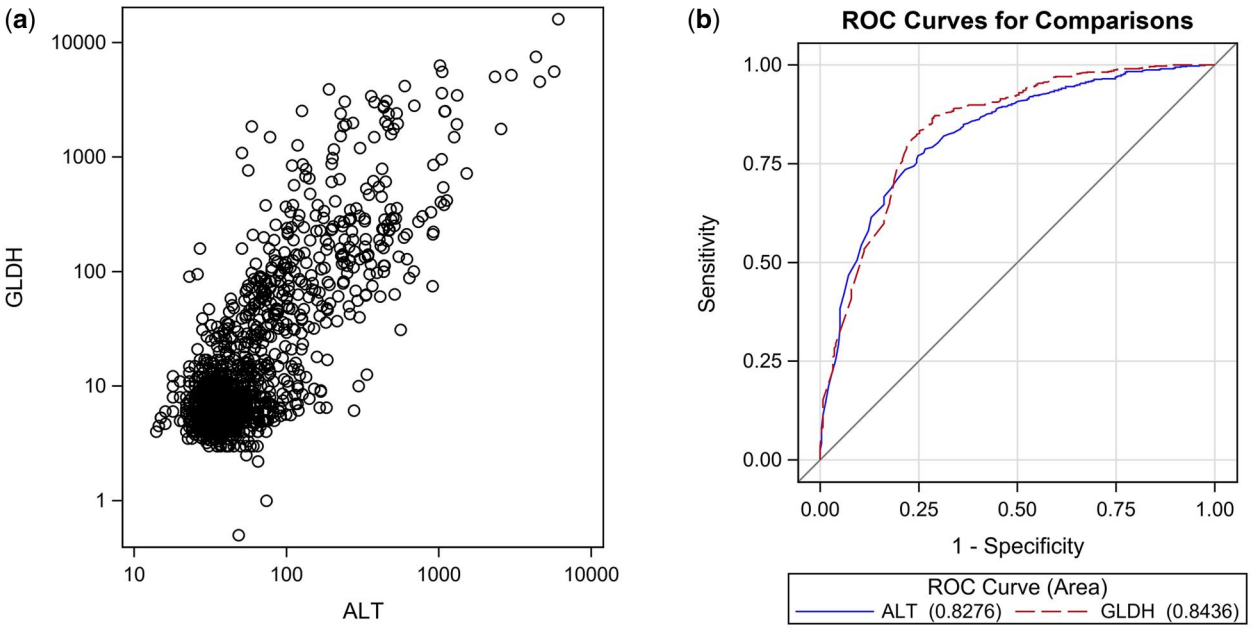

Fig. 3. Performance of GLDH to detect liver injury in rats treated with model toxicants. a) The data represent individual serum GLDH and ALT values measured in 877 rats treated with 30 model toxicants across 29 studies. b) The performance of serum GLDH to detect liver injury was compared with serum ALT using ROC analysis.

underlying muscle diseases (Schomaker et al. 2020). This is due to the fact that, unlike ALT, GLDH is not expressed in myocytes (Braakman et al. 1991; Kasarala and Tillmann 2016). However, since an appreciable amount of GLDH is expressed in the kidneys, pancreas, and intestine (Schmidt and Schmidt 1988; Mastorodemos et al. 2005), we next sought to evaluate the potential interference of kidney, pancreas, and GI disease in the detection of liver damage using GLDH. We evaluated serum GLDH levels in 237 subjects with adjudicated pancreatic, GI, or kidney injuries and compared them with serum GLDH in healthy subjects. To confirm the lack of interference of muscle injury on serum GLDH, we also examined samples from an additional 128 subjects with adjudicated muscle injury. We used the false positive rate (FPR) to assess potential confounding effects of organ injuries expressing GLDH on serum GLDH by calculating the percentage of subjects with GLDH values exceeding ULN (10 U/l), 2.5× ULN (25 U/l), and 5× ULN (50 U/l).

Among the 237 subjects with adjudicated kidney, pancreas, or GI injury (Table 2), we found only one subject (GLDH = 13 U/l) with serum GLDH greater than the ULN (10 U/l), yielding an FPR of only 0.4%. The analysis of 128 samples from subjects with muscle injury revealed that 12 subjects had serum GLDH above the ULN, including 2 subjects with levels above 2.5× ULN. This resulted in an FPR of 9.4% for subjects above the ULN and 1.6% for subjects exceeding the first serum GLDH level of concern 2.5× ULN. The adjudication of the absence of hepatocellular injury in subjects with underlying muscle impairments using serum chemistries and medical records is complicated by the serum ALT interference and might lead to underdiagnosing of hepatocellular injury.

Assessment of elimination kinetics of serum GLDH

Understanding the elimination kinetics of serum GLDH and their relationship to serum ALT in human subjects is essential for the interpretation of serum GLDH in the context of the time course of hepatocellular injury. We therefore calculated the elimination halftime ($T_{1/2}$) of serum GLDH and serum ALT in subjects with accidental APAP overdose. In total, 33 subjects who recovered from APAP overdose had sufficient serum GLDH and ALT time course values for inclusion in the analysis. In addition, we also calculated $T_{1/2}$ of GLDH and ALT in a previously described lethal case (Krauskopf et al. 2020). In all cases, the serum GLDH decreased about twice as fast as ALT (median $T_{1/2}$ ratio ALT/GLDH = 1.8 to 2.1). Although there was considerable variability in $T_{1/2}$ values for both GLDH and ALT, which can be attributed to differences in individual cases of accidental APAP overdose and potentially also to differences in the rate of recovery, the median $T_{1/2}$ values for serum GLDH and serum ALT in patients that recovered from APAP overdose were 22 and 40 h, respectively (Table 3). Furthermore, the median $T_{1/2}$ values of serum GLDH and ALT in the lethal case of APAP poisoning were consistent with $T_{1/2}$ values from recovering patients with $T_{1/2}$ ALT/GLDH ratios 2.1 and 1.8, respectively.

Discussion

Serum ALT is the gold standard biomarker of hepatocellular injury. However, persistent transaminasemia misdiagnosed as liver disease leads to delayed diagnosis of neuromuscular diseases due to unnecessary and invasive follow-up examinations. Therefore, the development of liver-specific biomarkers capable of detecting hepatocellular injury in patients with muscle injury is a critical unmet medical need.

Several strategies to detect hepatocellular injury in patients with muscle damage have been proposed. For example, the deviation of measured ALT levels from predicted ALT levels determined by the correlation relationship with muscle-specific CK is considered indicative of hepatocellular injury (Edge et al. 2006; Mathur et al. 2014). However, the natural variability of ALT and CK levels in subjects with muscle injury makes this approach difficult. In the search for liver-specific biomarkers, several groups proposed serum gamma-glutamyl transferase (GGT). Although GGT levels are unaffected by muscle diseases (Rosales et al.

Table 2. Assessment of liver specificity of serum GLDH in human subjects.

Group	Total	GLDH≤ULN	ULN<GLDH≤2.5× ULN	2.5× ULN<GLDH≤5× ULN	GLDH >5× ULN
Muscle	128	116 (90.6%)	10 (7.8%)	2 (1.6%)	0 (0%)
GI/pancreas/kidney	237	236 (99.6%)	1 (0.4%)	0 (0%)	0 (0%)
Group	**Total**	**ALT≤ULN**	**ULN<ALT≤3× ULN**	**3 ULN<ALT≤5× ULN**	**ALT >5× ULN**
Muscle	128	75 (58.6%)	40 (31.3%)	9 (7.0%)	4 (3.1%)

The values represent the number of subjects in individual category with false positive rate (FPR) in parenthesis.

Table 3. Assessment of elimination kinetics of serum GLDH compared with ALT in patients with APAP overdose.

	All recovered cases		
	GLDH (h)	ALT (h)	Half-life ratio (ALT/GLDH)
$T_{1/2}$ geometric mean	22	40	1.8
N	33	33	
		Lethal case	
$T_{1/2}$	17.2	35.6	2.1
N	1	1	

2008), the known poor performance of GGT for detection of hepatocellular damage—including the complete lack of sensitivity to detect hepatocellular injury in cases of acetaminophen poisoning—precludes the use of GGT for this purpose (Goldberg 1980; Robles-Diaz et al. 2015; Krauskopf et al. 2020). More recently, we proposed serum GLDH as a liver-specific alternative to ALT (Schomaker et al. 2020).

Serum GLDH is a promising biomarker capable of detecting drug-induced hepatocellular injury (Aubrecht et al. 2013; Roth et al. 2020). Because of its localization within the mitochondrial matrix, GLDH has been proposed as a mechanistic biomarker of mitochondrial damage (McGill et al. 2012, 2014), though that idea remains controversial (Church et al. 2020; McGill and Jaeschke 2021). It has also been proposed for use as a prognostic marker in acute liver failure (McGill et al. 2014) and drug-induced hepatocellular injury (Church et al. 2019). Like ALT, GLDH is released from injured hepatocytes following acute hepatocellular injury (Schmidt and Schmidt 1988; Kretzschmar et al. 2003; Harrill et al. 2012; Antoine et al. 2014). Since only a trace amount of GLDH is expressed in myocytes, muscle injury does not affect serum GLDH and it could serve as a liver-specific alternative to ALT and AST (Thulin et al. 2014; Schomaker et al. 2020). Nevertheless, the lack of reference intervals, defined thresholds of concern for hepatocellular injury, unknown interference of GLDH from extrahepatic tissues, and the lack of understanding of GLDH clearance kinetics limit the application of GLDH as a liver-specific biomarker of hepatocellular injury. Thus, we set out to define those parameters in the present study.

Reference intervals in healthy human populations are essential for the interpretation of biomarker data. We evaluated serum GLDH levels across a series of studies that included 959 healthy subjects and with serum ALT. All parameters, including mean, median, and ULN for both serum GLDH and serum ALT, were consistent across all studies. The combined ULN for serum GLDH was within the range of 7 to 11 U/l. This is in agreement with published studies (Kravos and Malešič 2008; Kravos and Malesic 2010; Kravos 2021). The serum ALT ULN from our combined dataset was within the range of 28 to 41 U/l. This is also in agreement with the literature (Kasarala and Tillmann 2016). As previously reported (Schomaker et al. 2013, 2020; Llewellyn et al. 2021), the serum GLDH proportionally mirrored serum ALT. In our study, the ULN of serum GLDH and ALT showed similar trends across ages, both having lower values in young (<6yo) and old (>61yo) age groups. Lower ALT levels in older and younger age groups were also observed in published studies (Ceriotti *et al.* 2010; Schwimmer *et al.* 2010; Sun *et al.* 2022; Vera *et al.* 2022). Close correlation between GLDH and ALT was observed when analyzing the effects of ethnic origin, with lower levels in the Asian population. On the other hand, sex had no effect on GLDH, whereas the serum ALT ULN was consistently lower in females than in males. The latter observation is consistent with prior literature on ALT (Ceriotti et al. 2010; Vera et al. 2022). Thus, GLDH may be a better marker than ALT across sexes.

To evaluate the diagnostic performance of serum GLDH to detect hepatocellular injury, we analyzed serum samples from subjects with a variety of chronic and acute liver diseases, including some with acetaminophen (APAP) overdose. We observed a tight correlation of serum GLDH with serum ALT with $r = 0.89$, in line with our previously published observations (Schomaker et al. 2013), and this correlation allowed us to determine thresholds of concern for GLDH, 2.5- and 5-fold over the ULN, that correspond to widely accepted serum ALT thresholds of 3- and 5-fold over the ULN. Thus, these thresholds could be used during clinical trials to indicate drug-induced hepatocellular injury. To further explore the relationship between GLDH and liver damage, we compared serum GLDH with microscopic hepatocellular lesions as a hepatocellular injury endpoint in rats treated with model hepatotoxicants. As in humans, serum GLDH is tightly correlated with ALT in rats as well as with the histological evidence of tissue damage.

The major advantage of serum GLDH is its potential to detect hepatocellular injury in subjects with underlying muscle damage (Schomaker et al. 2020). Indeed, the absence of muscle injury interference was confirmed using a separate cohort of patients with a variety of acute and chronic muscle diseases (Table 2). In the cohort of 128 subjects with muscle injury, only 12 subjects had serum GLDH above the ULN and only 2 had levels above 2.5×ULN. The incidence of patients with higher levels of serum GLDH in the cohort with muscle diseases can be explained by the patient selection criteria that relied on biomarkers that make diagnosis of mild liver impairment impossible due to the lack of liver-specific biomarkers of hepatocellular injury. The extrahepatic expression of GLDH is limited to the pancreas, kidney, and GI tissues (Schmidt et al. 1967; Schmidt and Schmidt 1988). Therefore, we assessed whether injury to these organs can interfere with serum GLDH levels. We analyzed GLDH levels in serum samples from 237 subjects with a variety of diseases of the pancreas, kidney, and GI tract. Only one subject out of 237 had serum GLDH above the ULN, demonstrating that elevated serum GLDH is specific to the liver and not affected by damage to other organs that express appreciable amounts of GLDH. Our studies demonstrate the utility of GLDH for diagnosing hepatocellular injury in subjects with acute muscle damage (Schomaker et al. 2020),

characterizing ALT increases associated with therapy (Harrill et al. 2012), monitoring liver safety in the clinical development of drugs for fatal neuromuscular diseases (Chowdhury et al. 2018; Wagner et al. 2021), and in studies assessing origin of ALT increases in absence of histopathological correlate (Harrill et al. 2014).

The relationship of biomarker response with the progression or recovery of tissue injury is essential for the interpretation of biomarker data. The serum GLDH in patients recovering from APAP overdose declines faster than serum ALT (Schomaker et al. 2013). This might be attributed to differences in elimination kinetics of GLDH ($T_{1/2}$=17 h) and ALT ($T_{1/2}$ = 42 h) reported by Schmidt and Schmidt (1988) and Schmidt et al. (1967). To investigate the elimination kinetics of these enzymes, we calculated $T_{1/2}$ in a set of 34 patients with APAP overdose including one fatal case. The median $T_{1/2}$ values for serum GLDH and ALT in our study (22 and 40 h for recovering patients and 17 and 35 h for the lethal case) were similar to $T_{1/2}$ values reported in the literature (Schmidt et al. 1967; Schmidt and Schmidt 1988). The similar $T_{1/2}$ between the nonlethal and lethal cases suggests that elimination of GLDH and ALT by Kupfer cells is not affected. The shorter $T_{1/2}$ of serum GLDH in comparison to ALT might be advantageous for assessing recovery and/or improving prognosis of acute hepatocellular injury caused by chemicals or drugs.

To our knowledge, our studies provide the most comprehensive evaluation of serum GLDH as a liver-specific biomarker of hepatocyte injury to date. In healthy subjects, the serum GLDH ULN (10 U/l) is stable and in contrast to ALT, it is not affected by gender. Since serum GLDH and ALT are tightly correlated, we linked the serum GLDH thresholds of concern (2.5× and 5× ULN) to ones of ALT (3× and 5× ULN). This is expected to significantly facilitate the interpretation of GLDH data. Applying these thresholds, we have shown that GLDH exhibits comparable performance to detect hepatocellular injury as serum ALT. The major advantage of GLDH over the gold standard ALT is its liver specificity and its ability to detect hepatocellular injury in subjects with underlying muscle impairments. Although more studies are needed, the shorter $T_{1/2}$ of GLDH than the one of ALT could also be advantageous for assessing recovery and/or prognosis of acute hepatocellular injury.

In conclusion, the presented data provide compelling evidence that serum GLDH should be considered as a liver-specific alternative to gold standard serum ALT that improves diagnosis of hepatocellular injury in subjects with muscle impairments, diagnosis of neuromuscular diseases, and liver safety monitoring in the development of drugs for muscle disorders.

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Supplementary material

Supplementary material is available at *Toxicological Sciences* online.

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