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Candidate Biomarkers for the Diagnosis and Prognosis of Drug-Induced Liver Injury: An International Collaborative Effort

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Current blood biomarkers are suboptimal in detecting drug-induced liver injury (DILI) and predicting its outcome. We sought to characterize the natural variabilty and performance characteristics of 14 promising DILI biomarker candidates. Serum or plasma from multiple cohorts of healthy volunteers (n = 192 and n = 81), subjects who safely took potentially hepatotoxic drugs without adverse effects (n = 55 and n = 92) and DILI patients (n = 98, n = 28, and n = 143) were assayed for microRNA-122 (miR-122), glutamate dehydrogenase (GLDH), total cytokeratin 18 (K18), caspase cleaved K18, glutathione *S*-transferase α, alpha-fetoprotein, arginase-1, osteopontin (OPN), sorbitol dehydrogenase, fatty acid binding protein, cadherin-5, macrophage colony-stimulating factor receptor (MCSFR), paraoxonase 1 (normalized to prothrombin protein), and leukocyte cell-derived chemotaxin-2. Most candidate biomarkers were significantly altered in DILI cases compared with healthy volunteers. GLDH correlated more closely with gold standard alanine aminotransferase than miR-122, and there was a surprisingly wide inter- and intra-individual variability of miR-122 levels among healthy volunteers. Serum K18, OPN, and MCSFR levels were most strongly associated with liver-related death or transplantation within 6 months of DILI onset. Prediction of prognosis among DILI patients using the Model for End-Stage Liver Disease was improved by incorporation of K18 and MCSFR levels. *Conclusion:* GLDH appears to be more useful than miR-122 in identifying DILI patients, and K18, OPN, and MCSFR are promising candidates for prediction of prognosis during an acute DILI event. Serial assessment of these biomarkers in large prospective studies will help further delineate their role in DILI diagnosis and management. (HEPATOLOGY 2019; 69:760-773).

Drug-induced liver injury (DILI) is a serious concern for patients, clinicians, and pharmaceutical companies, accounting for over half of the acute liver failure cases observed in Western countries.[1,2] Current detection and assessment of DILI relies on measurement of analytes that have been used for decades. Serum enzyme activities of aminotransferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and alkaline phosphatase (ALP) are quantified as measures of hepatocellular or cholestatic injury, respectively, and serum total bilirubin (TBIL) concentration is frequently used to assess global liver function. However, alterations in these biomarkers are not mechanistically informative, can occur for a variety of reasons unrelated to hepatic injury,[3-5] and can be observed with drugs that do not

Abbreviations: AFP, alpha-fetoprotein; AI, apoptotic index; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APAP, acetaminophen; ARG1, arginase-1; AST, aspartate aminotransferase; AUC, area under the curve; ccK18, caspase cleaved cytokeratin 18; CI, confidence interval; DILI, drug-induced liver injury; DILIN, Drug-Induced Liver Injury Network; FABP1, fatty acid binding protein-1; GLDH, glutamate dehydrogenase; GSTα, glutathione S-transferase α; INR, international normalized ratio; K18, total cytokeratin 18; LECT2, leukocyte cell-derived chemotaxin 2; LLoQ, lower limit of quantification; MCSFR, macrophage colony-stimulating factor receptor; MELD, Model for End-Stage Liver Disease; miR-122, microRNA-122; OPN, osteopontin; PSTC, Predictive Safety Testing Consortium; ROC, receiver operator characteristic; SAFE-T, Safer and Faster Evidence-Based Translation Consortium; SDH, sorbitol dehydrogenase; TBIL, total bilirubin; ULN, upper limit of normal.

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have the potential to cause clinically significant DILI.[6,7] They also are not specific or selective for DILI versus other causes of liver injury. Moreover, it is not currently possible to distinguish benign liver chemistry elevations from those that could lead to liver failure. Hence, even mild aminotransferase elevations can increase liver safety concern, especially in early clinical trials. A combination of an injury marker (aminotransferases) and a functional marker (TBIL) in the absence of cholestasis and when other causes have been excluded (i.e., Hy's Law case criteria) is a widely accepted prognostic model for DILI outcome.[8] However, most of the patients that meet Hy's Law case criteria will not require liver transplantation nor will they die. Ongoing efforts are exploring methods to improve severity prediction using traditional tests.[9] Nevertheless, there is a clear unmet need for biomarkers that are mechanistically informative and sensitive, as well as specific for prediction of DILI progression or resolution.

Substantial resources have been committed to understanding DILI and advancing candidate biomarkers that add value to traditional liver tests. Particularly, the Critical Path Institute's Predictive Safety

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Testing Consortium (PSTC) in the United States and the Safer and Faster Evidence-Based Translation (SAFE-T) consortium within the Innovative Medicines Initiative in Europe were leading major efforts to validate and qualify novel DILI biomarkers.[10] The Drug-Induced Liver Injury Network (DILIN) is a multicenter network in the United States created to prospectively biobank blood and tissue specimens from patients who have experienced DILI.[11] Each subject in this registry has undergone an unprecedented degree of phenotyping and most have at least 6-month follow-up data aiding assessments of long-term outcomes and prognosis. Given the overlapping goals of these three organizations, a cross-functional collaboration was established to study performance characteristics of candidate DILI biomarkers.

Candidate DILI biomarkers have been identified in preliminary evaluations. However, clinical application of these candidate markers requires robust performance for DILI detection and prognosis compared with current standards. Furthermore, it is critical that normal reference intervals be established for these biomarkers against which data from patients can be measured. We herein present the results from an international collaborative effort between PSTC, SAFE-T, and DILIN. Alpha-fetoprotein (AFP), arginase-1 (ARG1), cadherin-5, fatty acid binding protein-1 (FABP1), glutathione *S*-transferase α (GSTα), total cytokeratin 18 (K18), caspase cleaved K18 (ccK18), macrophage colony-stimulating factor receptor (MCSFR), osteopontin (OPN), glutamate dehydrogenase (GLDH), leukocyte cell-derived chemotaxin 2 (LECT2), paraoxonase-1 (normalized to prothrombin protein), sorbitol dehydrogenase (SDH), and microRNA-122 (miR-122) were assayed in serum or plasma from two cohorts of normal healthy subjects, two cohorts of patients that safely took potentially hepatotoxic drugs, and three cohorts of DILI patients (Table 1). DILI cohorts included patients who at 6 months had recovered completely, had persistent DILI or who died or required a liver transplant due to the DILI event within 6 months of DILI-onset.

Materials and Methods

HUMAN SUBJECT SAMPLE COLLECTION

Demographic data for the respective population cohorts can be found in Supporting Tables S1-3. All studies were conducted in accordance with protocols approved by local Institutional Review Boards, and written consent was received from all participants. Inclusion and exclusion criteria for each cohort are described in more detail in Supporting Methods. The primary causative drug implicated for all DILI patients is listed in Supporting Table S4. Following isolation of serum or plasma, samples were biobanked at −80°C until analysis and may have been frozen for multiple years.

BIOMARKER ANALYSIS

The 14 candidate biomarkers that were quantified in this study are listed in Table 1. Detailed methods for biomarker quantification can be found in the Supporting Methods. Briefly, serum or plasma traditional biomarker levels were quantified by clinical chemistry at the local institutions. Serum or plasma candidate biomarker levels were quantified in assays designed by or optimized by Natural and Medical Sciences Institute (Reutlingen, Germany) or at contract laboratories. Detailed information regarding validation parameters for all assays used in this study is provided in Supporting Table S5.

STATISTICAL ANALYSIS

Descriptive statistics, median and interquartile range, were used to describe continuous variables, and frequency and percent were used to describe categorical variables. All statistical analyses were performed using JMP Genomics version 8.1 or SAS software (Cary, NC) or GraphPad Prism 7.01 (La Jolla, CA). Biomarker distribution was visualized and the majority of displayed a log normal distribution. For consistency, the absolute value of all biomarkers, with the exception of the apoptotic index (AI), were log-transformed for statistical analyses. Statistical significance was considered $P < 0.05$.

REFERENCE INTERVAL DETERMINATION

PSTC healthy volunteer data and SAFE-T Tel Aviv healthy volunteer data were analyzed for the determination of reference intervals. The reference interval lower limit of normal and upper limit of normal (ULN) was defined by the 5th and 95th percentile of the population, respectively, using a mixed-model approach and fitted a random subject term. For two of the markers (ccK18 and GSTα) a substantial number of values were below the lower limit of

TABLE 1. Candidate Biomarkers

Candidate Biomarker	Physiological Function	Tissue Localization	Potential Utility in DILI	Cohorts Analyzed
AFP	Plasma protein thought to be the fetal form of albumin	High levels in liver progenitor cells	Regeneration (progenitor cells)	DILIN, PSTC, SAFE-T
ARG1	Hydrolase enzyme that catalyzes the hydrolysis of arginine to urea and orthinine	High levels in liver; lower levels in erythrocytes, kidney and brain	Cell injury/death	DILIN, PSTC, SAFE-T
CDH5	Calcium-dependent transmembrane adherens junction protein important for endothelial cell integrity and cell–cell adhesion	Broad localization including liver	Susceptibility	DILIN, PSTC, SAFE-T
FABP1	Protein involved in binding and transport of fatty acid	High levels in the liver; lower levels in kidney and gastrointestinal tract	Cell injury/death	DILIN, PSTC, SAFE-T
GSTα	Phase 2 detoxification enzyme that catalyzes the conjugation of glutathione with various electrophiles	High levels in the liver and multiple tissues	Cell injury/death	DILIN, PSTC, SAFE-T
K18/ccK18	Type 1 intermediate protein expressed in epithelial cells responsible for cell structure and integrity; caspase cleavage results in a fragmented form of protein (ccK18)	Broad localization including liver	Cell injury/death, mechanism	DILIN, PSTC, SAFE-T
MCSFR	Receptor on macrophages/monocytes for CSF, a cytokine that controls the proliferation, differentiation, and function of macrophages	Broad localization including liver	Inflammation	DILIN, PSTC, SAFE-T
OPN	Phosphoprotein involved in migration/infiltration of inflammatory and cancer cells	Broad localization including liver	Inflammation, regeneration (progenitor cells)	DILIN, PSTC, SAFE-T
GLDH	Mitochondrial matrix protein that catalyzes the conversion of 2-oxoglutarate to L-glutamate	High levels in the liver; lower levels in kidney and brain	Cell death, mechanism	PSTC, SAFE-T
LECT2	Protein involved in the recruitment of neutrophils	High expression in the liver; lower expression in the testis	Regeneration (hepatocytes)	PSTC, SAFE-T
PON1	HDL-associated enzyme that participates in paraoxonase, arylesterase, and dyazoxonase activites; useful in diagnosis of NAFLD and NASH when normalized to prothrombin	Produced in liver, released constitutively into circulation	Function	PSTC, SAFE-T
SDH	Enzyme involved in carbohydrate metabolism that converts sorbitol into fructose	High levels in the liver, kidney, and testis; lower levels in multiple tissues	Cell injury/death	PSTC, SAFE-T
miR-122	Liver-specific microRNA that posttranscriptionally regulates mRNA involved in processes including hepatocyte differentiation and lipid/cholesterol metabolism	High levels in the liver	Cell injury/death	PSTC, SAFE-T

Abbreviations: AFP, alpha-fetoprotein; ARG1, arginase-1; ccK18, caspase cleaved cytokeratin 18; CDH5, cadherin-5; CSF, colony-stimulating factor; DILI, drug-induced liver injury; DILIN, Drug-Induced Liver Injury Network; FABP1, fatty acid binding protein-1; GLDH, glutamate dehydrogenase; GST-α, glutathione *S*-transferase α; HDL, high-density lipoprotein; K18, cytokeratin 18; LECT2 leukocyte cell-derived chemotaxin 2; MCSFR, macrophage colony-stimulating factor receptor; miR-122, microRNA-122; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OPN, osteopontin; PON1, paraoxonase-1 (normalized to prothrombin protein); PSTC, Predictive Safety Testing Consortium; SAFE-T, Safer and Faster Evidence-Based Translation; SDH, sorbitol dehydrogenase.

quantification (LLoQ), so a maximum likelihood estimate for a truncated log-normal distribution was used to estimate the ULN. Because approximately 90% of the data for K18 were below the LLoQ, only the ULN was calculated by a nonparametric method. PSTC collected three serial biomarker measurements in all subjects and a mixed model was used to obtain the variance components for inter- and intra-individual variation assuming log-normal distribution. The reference interval was obtained using the estimated mean and standard deviation for the log-normal distribution.

TABLE 2. Biomarker Reference Intervals in Healthy Volunteers

			PSTC (n = 81)*					SAFE-T (n = 192)			
				LLN	ULN				LLN	ULN	
Biomarker	Unit	Matrix	Estimated Geometric Mean	Estimated 5th Percentile	Estimated 95th Percentile	Intersubject % CV	Intrasubject % CV	Estimated Geometric Mean	Estimated 5th Percentile	Estimated 95th Percentile	Intersubject % CV
AFP	ng/mL	Serum	0.68	0.24	1.98	61.53	31.93	0.99	0.28	3.54	90.21
ARG1	ng/mL	Serum	7.63	3.00	19.46	46.03	37.46	35.97	18.38	70.38	42.57
ccK18	U/L	Serum	90.65	31.59	260.16	70.97	34.76	139.99	52.46	373.55	65.39
CDH5	ng/mL	Serum	2798.89	1853.77	4225.87	18.00	17.69	2287.79	1222.52	4281.33	39.52
FABP1	ng/mL	Serum	6.91	3.29	14.55	32.75	32.86	9.21	4.57	18.54	44.55
GLDH	U/L	Serum	2.71	1.01	7.24	52.74	34.53	3.00	0.95	9.51	79.68
GSTα	ng/mL	Serum	6.31	0.68	60.00	119.54	71.86	6.61	0.71	64.11	172.57
LECT2	ng/mL	Plasma	252.27	142.07	447.96	28.64	20.97	177.96	84.74	373.74	47.50
miR-122	copies/μL	Serum	2152.98	347.05	13356.52	90.89	93.56	3173.64	368.02	27367.61	213.51
MCSFR	ng/mL	Plasma	334.81	196.1	571.64	30.08	13.89	306.67	175.98	534.39	34.75
OPN	ng/mL	Serum	4.13	1.66	10.31	52.15	26.61	6.54	2.68	15.99	58.56
PON1	ng/μg	Plasma	4.91	2.02	11.93	44.16	34.57	9.44	4.18	21.35	52.81
SDH	U/L	Serum	3.02	1.18	7.75	43.43	41.01	1.79	0.79	7.17	101.57
K18†	U/L	Serum			121.35					151.14	

Abbreviations: AFP, alpha-fetoprotein; ARG1, arginase-1; ccK18, caspase cleaved cytokeratin 18; CDH5, cadherin-5; CI, confidence interval; CV, coefficient of variation; FABP1, fatty acid binding protein-1; GLDH, glutamate dehydrogenase; GSTα, glutathione S transferase alpha; K18, cytokeratin 18; LECT2, leukocyte cell derived chemotaxin 2; LLN, lower limit of normal; MCSFR, macrophage colony-stimulating factor receptor; miR-122, microRNA-122; OPN, osteopontin; PON1, paraoxonase-1 (normalized to prothrombin protein); PSTC, Predictive Safety Testing Consortium; SAFE-T, Safer and Faster Evidence-Based Translation; SDH, sorbitol dehydrogenase; ULN, upper limit of normal.
*Three serial collections were collected for each individual. The mean value for each individual was used for all statistical analyses with the exception of intraindividual % CV.
†90% of K18 data was below the lower limit of quantification, therefore only an upper reference interval was determined.

BIOMARKERS OF DILI DETECTION

Receiver operator characteristic (ROC) curve analysis was utilized to determine the performance of traditional and candidate biomarkers for detection of DILI patients. Biomarkers were considered predictive of DILI if both the ROC area under the curve (AUC) and the lower end of the 95% confidence interval (CI) >0.5. All healthy volunteer and patient datasets (PSTC, SAFE-T, and DILIN) were used for this analysis. The relationship of liver-specific GLDH and miR-122 to ALT was examined. Correlation of GLDH and miR-122 with ALT was determined using Pearson's *r* coefficient.

BIOMARKERS OF DILI PROGNOSIS

Accurate outcome assessments were available only for the DILIN subjects. ROC curve analysis

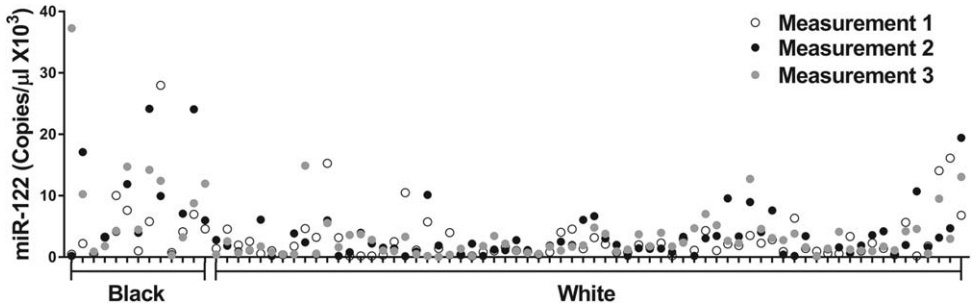

FIG. 1. Intraindividual variabilty in PSTC cohort observed in miR-122 quantifications. Three fasting blood samples were collected from volunteers over the course of 21 days. Greater intrasubject variability was observed in miR-122 levels among black subjects in this study compared with white subjects. Each bar represents an individual subject; circles represent data for miR-122 measurements 1 (white), 2 (black), and 3 (gray).

was used to determine which biomarkers measured in the initial DILIN sera could significantly predict which patients died/required a liver transplantation or developed unresolved DILI. For a detailed description of this analysis, refer to the Supporting Methods.

Results

BIOMARKER LEVELS IN HEALTHY VOLUNTEERS

The natural variation of candidate biomakers was explored in two cohorts of healthy volunteers (Supporting Tables S1 and S2). Multiple samples returned values below the LLoQ for AFP, ccK18, GSTα, K18, SDH, and miR-122. The geometric mean, intersubject variation and reference interval (5th and 95th percentiles) for each biomarker are presented in Table 2. Because of the large number of samples with K18 values that fell below the LLoQ, only the estimated 95th percentile was calculated for this biomarker. In both healthy volunteer cohorts, intersubject variability of miR-122 was also high [% coefficient of variation of 90.89 and 213.51 in the PSTC and SAFE-T cohorts, respectively]. Furthermore, miR-122 also showed substantial intrasubject variability in the PSTC cohort (intrasubject % coefficient of variation of 93.56), and this appeared to be most prominent among black individuals (Fig. 1).

The biomarker reference intervals between PSTC and SAFE-T showed substantial overlap, although the geometric mean tended to be slightly higher in the SAFE-T cohort. ARG1 levels, however, were considerably increased in the SAFE-T cohort, compared with the PSTC cohort.

BIOMARKER PERFORMANCE FOR DETECTING DILI

All candidate DILI biomarkers significantly identified patients with DILI with the exception of LECT2 (Table 3). The lower CI limit of LECT2 was only 0.45, indicating that the data cannot rule out random agreement between predictions and outcome. K18, ccK18, FABP1, and GLDH, had AUCs > 0.9, indicating that these biomarkers are the most accurate candidate biomarkers for the detection of DILI.

GLDH levels showed a very strong correlation with ALT levels ($r = 0.88$, $P < 0.0001$; Fig. 2A). miR-122 levels were also significantly correlated with levels of ALT, although the strength of the correlation was reduced compared with that for GLDH ($r = 0.66$; $P < 0.0001$; Fig. 2B).

TABLE 3. Biomarker Performance in DILI Identification*

Category	Biomarker	AUC	95% CI
Traditional	ALT	0.990	0.984-0.996
Traditional	AST	0.975	0.963-0.987
Traditional	ALP	0.902	0.873-0.930
Traditional	TBIL	0.857	0.821-0.892
Candidate	K18	0.947	0.928-0.966
Candidate	FABP1	0.916	0.890-0.941
Candidate	ccK18	0.911	0.887-0.935
Candidate	GLDH	0.907	0.870-0.945
Candidate	MCSFR†	0.854	0.822-0.887
Candidate	miR-122	0.831	0.779-0.883
Candidate	AFP	0.826	0.793-0.859
Candidate	GSTα	0.827	0.792-0.862
Candidate	SDH	0.819	0.763-0.876
Candidate	OPN	0.758	0.718-0.799
Candidate	CDH5	0.658	0.614-0.701
Candidate	PON1	0.612	0.542-0.682
Candidate	ARG1	0.564	0.519-0.609
Candidate	LECT2	0.519	0.450-0.588

Abbreviations: AFP, alpha-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ARG1, arginase-1; AST, aspartate aminotransferase; AUC, area under the curve; ccK18, caspase cleaved cytokeratin 18; CDH5, cadherin 5; CI, confidence interval; DILI, drug-induced liver injury; DILIN, DILI Network; FABP1, fatty acid binding protein-1; GLDH, glutamate dehydrogenase; GST-α, glutathione S transferase alpha; K18, cytokeratin 18; LECT2, leukocyte cell derived chemotaxin 2; MCSFR, macrophage colony-stimulating factor receptor; miR-122, microRNA-122; OPN, osteopontin; PON1, paraoxonase-1 (normalized to prothrombin protein); PSTC, Predictive Safety Testing Consortium; SAFE-T, Safer and Faster Evidence-based Translation; SDH, sorbitol dehydrogenase; TBIL, total bilirubin.
*Statistical data for all biomarkers were calculated using patient data from PSTC healthy volunteers (n = 81), SAFE-T healthy volunteers (n = 192), SAFE-T subjects that safely received DILI-eliciting compounds (n = 147), and SAFE-T DILI patients (n = 126). DILIN patient data (n = 143) were also used for all biomarkers with the exception of GLDH, miR-122, SDH, PON1, and LECT2.
†DILIN measurements were collected in serum; all other measurements were collected in plasma.

BIOMARKER ALTERATIONS BY DRUG CLASS

The SAFE-T DILI cohorts contain data from patients with acetaminophen (APAP)-related liver injury, as well as from patients who experienced idiosyncratic DILI related to various compounds (Supporting Table S4). To determine whether one or more DILI compounds/classes produces signature biomarker changes that are unique compared with APAP-related hepatotoxicity, SAFE-T DILI patient data were divided into broad drug classes

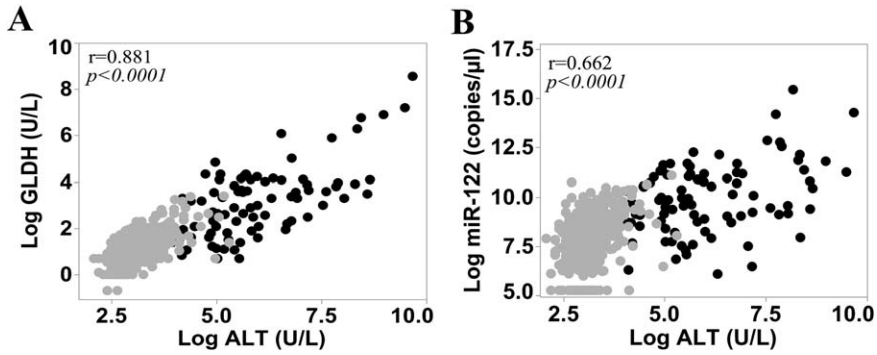

FIG. 2. Correlation between levels of ALT and liver-specific biomarkers (A) GLDH and (B) miR-122. Data points are individual PSTC and SAFE-T subject samples and represent individuals that did not have DILI (gray) and individuals that did have DILI (black). Values are log normalized. Pearson's *r* coefficient is shown.

(Supporting Methods). In general, biomarkers (including ALT) tended to be the most altered from other drug classes in APAP-related hepatotoxicity, emphasizing the acute and severe injury this compound causes (Supporting Fig. S1A). However, several biomarkers were the most elevated in flupirtine DILI. Specifically, TBIL ($P < 0.05$ versus APAP, antibiotics, chemotherapy, nonsteroidal anti-inflammatory drugs, other [Supporting Fig. S1B]), cadherin-5 ($P < 0.05$ versus APAP, antibiotics, chemotherapy, other [Supporting Fig. S1C]), and MCSFR ($P < 0.05$ versus APAP, antibiotics, chemotherapy, nonsteroidal anti-inflammatory drugs, other [Supporting Fig. S1D]) were significantly elevated in flupirtine-related liver injury. Flupirtine is an aminopyridine used as a nonopioid analgesic that is well recognized to cause serious DILI in Europe (it is not available in the United States); all SAFE-T patients with flupirtine-related liver injury met Hy's Law case criteria. When DILIN data were divided into drug categories, no significant differences were observed; however, DILIN data does not have patients with either APAP- or flupirtine-related hepatotoxicity (data not shown).

Differences between cohorts in amoxicillin with clavulanic acid (Augmentin) DILI were explored. We found that DILIN patients with Augmentin-induced hepatotoxicity had significantly elevated levels of ALT, ARG1, FABP1, GST-α, K18, and ccK18 ($P < 0.05$ for all) compared with SAFE-T DILI patients (Supporting Table S6).

BIOMARKER PERFORMANCE AS PROGNOSTIC MARKERS

Death/Transplantation

The DILIN samples used in the present study were limited to those collected within 2 weeks of DILI onset and, per protocol, patients were tracked for at least 6 months. A small subset of DILIN patients died/required liver transplantation (n = 15) within 6 months and it was determined that the DILIN event was the cause.[12] ROC curve analysis demonstrated that traditional biomarkers including the international normalized ratio (INR), AST, and TBIL were predictive of death/liver tranplantation (lower 95% CI limit > 0.5; Table 4). Of the subset of candidate biomarkers measured in the DILIN dataset (Table 1), elevated

TABLE 4. Prognostic DILI Biomarkers for Death/Liver Transplantation

Category	Biomarker	AUC	95% CI	Value at Youden's J*
Traditional	INR	0.920	0.864-0.977	0.47
Traditional	TBIL	0.821	0.733-0.909	5.57
Traditional	AST	0.7	0.587-0.814	5.05
Traditional	ALT	0.606	0.433-0.78	6.68
Traditional	ALP	0.597	0.433-0.76	5.01
Candidate	OPN	0.858	0.759-0.957	3.38
Candidate	K18	0.832	0.737-0.927	7.98
Candidate	MCSFR	0.775	0.654-0.896	6.94
Candidate	ccK18	0.778	0.676-0.881	6.96
Candidate	FABP1	0.721	0.608-0.833	4.21
Candidate	AFP	0.687	0.566-0.809	1.57
Candidate	CDH5	0.623	0.498-0.748	8.01
Candidate	ARG1	0.588	0.436-0.741	3.47
Candidate	GST-α	0.536	0.359-0.713	6.88
Candidate	AI	0.761	0.627-0.895	0.37

Abbreviations: AFP, alpha-fetoprotein; AI, apoptotic index; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ARG1, arginase-1; AST, aspartate aminotransferase; AUC, area under the curve; ccK18, caspase cleaved cytokeratin 18; CDH5, cadherin-5; CI, confidence interval; FABP1, fatty acid binding protein-1; GST-α, glutathione *S*-transferase alpha; INR, international normalized ratio; K18, cytokeratin 18; MCSFR, macrophage colony-stimulating factor receptor; OPN, osteopontin; TBIL, total bilirubin.
*All values with the exception of AI are log-normalized. Youdin's J is a statistic that estimates the probability of an informed decision.

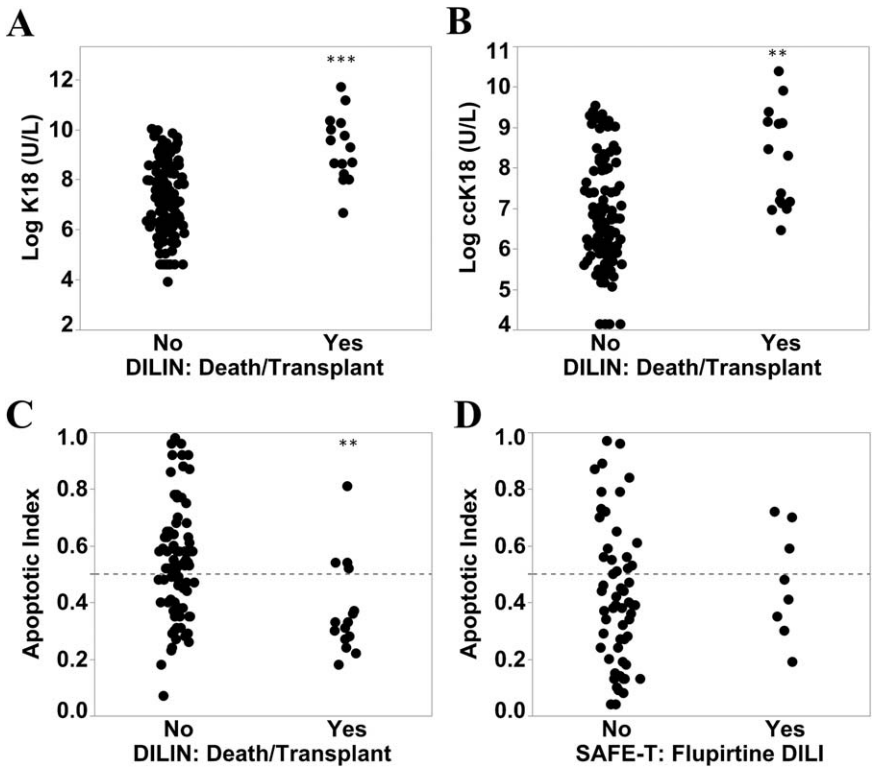

FIG. 3. Assessment of K18 measurements. Differences in serum (A) K18, (B) ccK18, and (C) AI between DILIN patients who did not die/require liver transplantation by 6 months after DILI onset and those that did. (D) Differences in AI between SAFE-T patients experiencing DILI associated with flupirtine utilization and patients experiencing DILI unrelated to flupirtine. Data points represent individual patients. A dotted line for AI is drawn at 0.5, representing a score that suggests an equal contribution of apoptosis and necrosis. Values for K18 and ccK18 are log-normalized. $^{**}P < 0.01$ and $^{***}P < 0.001$.

levels of OPN, K18, MCSFR, ccK18, FABP1, and AFP significantly predicted death/transplantation. INR was found to have the strongest association with death/transplantation (AUC = 0.920) closely followed by OPN (AUC = 0.858). The predictive biomarkers had more than 2-fold and more than 7-fold changes over DILI patients that did not experience liver failure and healthy volunteers, respectively (Supporting Table S7).

The values of K18 and ccK18 measured in 98 DILIN patients enabled the calculation of an AI (see Supporting Methods for details) and AI (ccK18:K18 ratio) was also explored as a prognostic biomarker of death/liver tranplantatino (Table 4). Although both K18 and ccK18 levels were elevated in patients who experienced death/liver transplantation compared with patients who did not, the AI was significantly reduced in patients who died/required liver transplantation (Fig. 3A-C). An AI was additionally calculated in 64 SAFE-T patients with DILI. Patients with flupirtine-related DILI did not have a significantly different mean AI compared with patients who had APAP-induced liver injury or DILI associated with other compounds (Fig. 3D).

Current prognostic models for liver transplantation and death were explored to identify whether incorporation of biomarkers that passed filtering criteria (OPN, K18, MCSFR, and AFP; see Supporting Methods for details) could improve prediction. Model for End-Stage Liver Disease (MELD) score $\geq$ 20 was highly sensitive and MELD score $\geq$ 30 was highly specific for prognosis of death/transplantation in the DILIN population. Incorporating K18 and MCSFR levels with MELD score (when MELD values were 20-29; Fig. 4) improved the specificity of using MELD score $\geq$ 20 (specificity of 0.889 when incorporating K18 and MCSFR with MELD score $\geq$20 versus 0.738 with MELD score $\geq$ 20 alone; Supporting Table S8) without reducing the sensitivity of using MELD score $\geq$ 20 alone (sensitivity of 0.933 for both). Hy's Law showed moderate performance for prediction of death

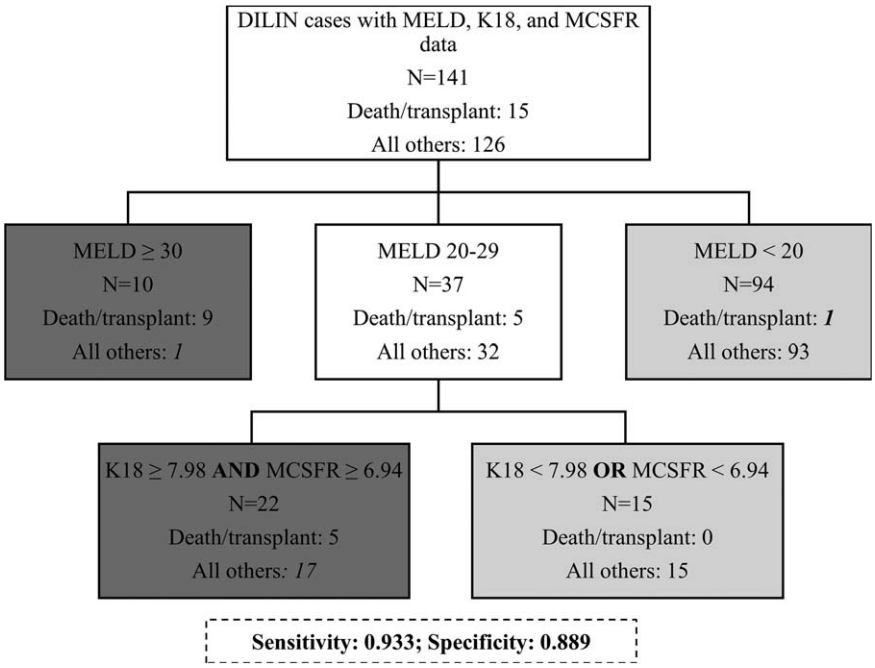

FIG. 4. Incorporation of candidate biomarkers into MELD score prognostic model. The model was optimized for prediction of death/transplantation in DILIN patients (n = 141) using the MELD score, total K18, and MCSFR measurements (sensitivity = 0.933, specificity = 0.889). White boxes represent branching points; light gray boxes represent patients not predicted to have an adverse outcome; and dark gray boxes represent patients predicted to die/require transplantation. Numbers in italics represent false results (i.e., 18 false positives and 1 false negative).

or liver transplant in this DILIN cohort (sensitivity of 0.8 and specificity of 0.634).

Unresolved DILI

Nineteen patients in the DILIN cohort had unresolved DILI at their 6-month follow-up visit (persistently elevated ALT, AST, ALP, or TBIL over ULN with no competing etiology). Consistent with previous data, only elevated levels of ALP predicted the outcome of these patients (lower CI limit > 0.5; Supporting Table S9).[13] Within this subset, six of the 19 patients with unresolved DILI had ALP levels that were elevated at their 6-month follow-up visit. When the data were reanalyzed to determine whether candidate biomarkers could predict this subset of patients, it was found that GST-α (measured within 2 weeks of DILI onset) was significantly lower in patients with prolonged ALP elevation (ROC AUC = 0.760, 95% CI = 0.509-1.0).

Discussion

The traditional biomarkers of DILI may not be adequately liver-specific, offer little mechanistic insight into mode of injury, and are not sufficiently prognostic for injury outcome/resolution. In the current study, the performance of 14 candidate DILI biomarkers was explored in cohorts of healthy volunteers, in patients who received known DILI-eliciting compounds without developing liver injury, and in patients who experienced DILI.

Most of the biomarker reference intervals showed sizeable overlap between the SAFE-T and PSTC healthy volunteer cohorts. ARG1 levels, however, were substantially higher in the SAFE-T volunteers compared with the PSTC volunteers (Table 2). This difference could not be accounted for by differences in racial diversity or age. Additionally, each biomarker was quantified at the same facility, with the same validated assays, making technical variability an unlikely explanation to account for this difference. However, biomarker stability at −80° should be explored as a possible explanation for ARG1 population differences, given that samples were stored from 3 months to 3 years. The influence of race (primarily whites versus African Americans) on biomarker reference ranges was explored in the PSTC cohort. Although small differences were observed (such as for ARG1), there was considerable overlap between biomarker ranges,

and unique reference intervals were deemed to be unnecessary in this small population.

The biomarkers examined in this study were selected based on preliminary performance data generated by SAFE-T in a small pilot cohort of DILI patients and healthy volunteers (data not shown). Therefore, it is not surprising that most of the candidate biomarkers were significantly elevated in DILI. In particular, K18, FABP1, GLDH, and ccK18 had ROC AUCs >0.90, suggesting that these biomarkers in particular may be useful when screening for DILI. The sensitivity of these biomarkers for the detection of DILI or hepatotoxicity, in general, is in agreement with previously reported data.[14-20] Biomarker differences in patients with Augmentin-induced heptotoxicity were explored between the DILIN and SAFE-T cohorts, and several candidate biomarkers were found to be elevated in the DILIN patients. DILIN patients also had higher mean elevations in serum ALT, therefore DILIN patients may in general have been sicker than SAFE-T patients. Indeed, whereas SAFE-T enrolled patients with ALT >3× ULN, DILIN inclusion criteria specifies that patients must have an ALT >5× ULN.

Although very large elevations in serum ALT are generally believed to be liver-specific, ALT elevations are also noted following strenuous exercise and in patients with muscle diseases such as muscular dystrophy.[3,21] A biomarker with greater liver specificity could be valuable in the clinic when the source of an ALT elevation is uncertain. GLDH and miR-122 are highly liver-specific and are not altered in response to muscle injury.[22,23] GLDH is a large protein found within the matrix of mitochondria, enriched in the liver.[24] The interindividual variability of GLDH ranged from approximately 53% in the PSTC cohort to approximately 80% in the SAFE-T cohort, and intraindividual variability was minimal (35%). In contrast, there was significant interindividual variability of miR-122, a liver-specific microRNA that makes up as much as 70% of hepatic microRNA content,[25] in both cohorts (∼91% and ∼213% in the PSTC and SAFE-T cohorts, respectively), and remarkable intraindividual variability, most evident among African Americans in the PSTC cohort, was also observed (∼94%).

miR-122, unlike other candidate biomarkers, may not simply be leaked passively from injured cells, although this is believed to be the primary mechanism following injury. Instead, evidence suggests that miR-122 can be released actively from the liver, at least in part within extracellular vesicles, in response to stress.[26] For example, it has recently been shown that in response to stimuli and in the absence of overt hepatocyte death, miR-122 can be released and can modulate activation of innate immune cells or directly regulate kidney release of erythropoetin.[27,28] Therefore, the variability that is inherent to miR-122 levels not only between individuals but also within the same individual likely results from physiologic processes unrelated to damage to the liver. The complexity of the assay (relative to other biomarker assays) and the lack of a universally accepted method for data normalization may also contribute to the variability observed. For these reasons, the PSTC has recently deprioritized its pursuit of miR-122 as a liver-specific biomarker in favor of GLDH, which is measured by routine clinical chemistry.

GLDH demonstrated enhanced correlation with ALT levels and improved performance for detection of DILI compared with miR-122. Schomaker et al.[18] explored GLDH as a biomarker of hepatocellular necrosis in patients with liver impairment and found it to be superior to other candidate biomarkers (miR-122 was not evaluated in that study). Other studies have also demonstrated that GLDH is elevated in patients with APAP-related toxicity.[29,30] Furthermore, GLDH has been proposed as a potentially early indicator of recovery from DILI due to the fast elimination of GLDH observed in subjects recovering from accidental APAP overdose with persisting high levels of ALT.[14]

It should be noted that in spite of the large inter- and intraindividual variations in serum levels of miR-122 that we report here, miR-122 appears to be useful in predicting liver injury after APAP overdose. Dear et al.[31] reported recently that miR-122 demonstrated the highest performance for prediction of APAP-induced acute liver injury in a large cohort of overdose patients with normal ALT levels at presentation, confirming results of an earlier study.[14] In contrast, GLDH was not useful in this context. Because the method for quantitation of miR-122 in these studies differed from ours, it is unclear whether the levels of miR-122 in patients susceptible to APAP injury were simply above the range of inter- and intraindividual variation measured in the healthy volunteers in our study. Although the assumption is that elevated serum levels of miR-122 reflect early hepatocyte stress or injury due to APAP, given the increasing appreciation of the physiological roles of miR-122, it is possible that individuals with high baseline serum levels of miR-122 are more susceptible to APAP injury.

The prognostic performance of GLDH and miR-122 was not determined in the current study because these biomarkers were not measured in the DILIN patient cohort (due to sample volume limitations) where outcome data was systematically collected. However, semiquantitative measurements of miR-122 have previously been conducted in a subset of DILIN patients.[32] In contrast to the data observed in APAP-induced hepatotoxicity, reduced serum levels of miR-122 and albumin were observed in patients that died within 6 months of DILI onset compared with patients who recovered. Collectively, these data suggest that both miR-122 and GLDH likely have utility in predicting and managing DILI and factors related to extent of injury at serum collection time and biomarker half-life may influence the interpretation of biomarker alterations. Research exploring the kinetics of these biomarkers in DILI may aid in interpretation of these biomarkers in the clinic.

Several biomarkers showed promise as prognostic biomarkers for death/transplantation in DILI. In particular OPN, K18, and MCSFR performed well as predictors of death/transplantation. Increased levels of each of these biomarkers were observed in DILIN death/transplantation patients compared with all others. OPN showed the best performance for prognosis of all candidate biomarkers in DILIN patients. OPN is associated with liver regeneration due to activation of hepatic stem cells.[33] While elevated levels of AFP, which is also released from hepatic stem cells, were prognostic for death/transplantation, the performance of this biomarker was reduced compared with OPN (AUC = 0.687 versus 0.858 for AFP and OPN, respectively).

The increase in OPN observed in this study is in contrast to recent data demonstrating that patients with acute liver failure (from various etiologies) who died/received a transplant had reduced levels of plasma OPN compared with those who recovered.[34] The difference between these studies may be related to the timing of sample collection. Entry into the previous study required acute liver failure to be occurring at enrollment (INR $\geq$ 1.5 and encephalopathy), suggesting more advanced injury than present in the current cohort.

MCSFR, another marker of inflammation, is among the most promising prognostic candidate biomarkers (AUC = 0.775) in the DILIN data for death/transplantation. MCSFR, the receptor for CSF-1, is thought to be shed from activated macrophages during DILI.[35] Interestingly, reduced levels of CSF-1 were associated with poor outcome in patients experiencing APAP-induced liver injury, and this was thought to suggest that macrophages and an innate immune response are necessary for regeneration following liver injury.[36] The cause of this discrepancy is unclear, but may be related to the type of DILI examined in the current study (idiosyncratic versus intrinsic). MCSFR levels were considerably higher in SAFE-T patients experiencing flupirtine-induced hepatotoxicity compared with the 19 cases of APAP-induced hepatotoxicity, despite ALT levels being markedly higher in APAP-induced liver injury (Fig. 2). Although no SAFE-T DILI patients died or required a liver transplant, all patients that experienced flupirtine-induced DILI met Hy's Law case criteria, suggesting that increased MCSFR may be indicative of severe idiosyncratic DILI (versus APAP-induced liver injury).

K18 also showed value as a prognostic biomarker in DILIN patients; K18 levels were elevated in patients who died or required liver transplantation compared with patients who survived. We found that incorporating K18 (threshold: log-normalized value of 7.98) into a model that stratified risk based on MELD score cutoffs of $\geq$20 and $\geq$30 improved specificity of using MELD $\geq$ 20 alone without decreasing sensitivity. The addition of MCSFR further improved the specificity, albeit slightly. The value of K18 for prediction models of death following hepatotoxicity is in agreement with previously published literature.[15,37]

K18 is an intermediate filament found in epithelial cells, including hepatocytes. Necrosis results in passive leakage of full length K18 into circulation while cleavage of K18 by caspases results in leakage of ccK18 into circulation following apoptotis.[38] Apoptosis is thought to be a more benign form of injury because apoptosis is not believed to result in the release of damage associated molecular pattens and subsequent activation of the innate immune system.[39,40] Determination of the ccK18/K18 ratio, the AI, is believed to reflect the proportion of cell death that can be attributed to apoptosis. DILIN patients who died or required liver transplantation had lower AIs than patients who recovered from DILI (i.e., consistent with necrosis as the predominate form of cell death in patients that died or required liver transplantation). Because biopsies are not routinely conducted in the clinic, validation of the AI in humans is challenging. Nevertheless, pilot data in DILIN patients suggest that the AI may be useful in predicting the degree of apoptosis vs necrosis in liver tissue.[41]

It should be noted that in the DILIN cohort, 2 weeks was the maximum time between DILI onset (the time at which a patient's serum liver chemistries first qualified for DILIN entry) and research blood collection, but the time between symptom onset (when known) and research blood collection varied to a larger degree (2-90 days). We were unable to detect significant correlations between biomarker levels and days between symptom onset and research blood collection ($r = -0.014$ to 0.222; Supporting Table S10). For example, the interval between symptom onset and determination of serum levels of K18 and MCSFR had Pearson's r coefficients of 0.142 and 0.163, respectively. Because variation in biomarker release and clearance kinetics may significantly affect the interpretation of biomarker levels, future studies should include serial samples collected over a broad range of intervals from symptom onset.

Resulting from the data presented here, both the US Food and Drug Administration and the European Medicines Agency issued letters of support that explicitly encourage the exploratory use of selected biomarkers in drug registration trials and further development of K18, OPN, and MCSFR as potential diagnostic or prognostic DILI biomarkers.[42,43] Further exploration of both miR-122 and GLDH as liver-specific alternatives to ALT was also encouraged by these regulatory agencies.

In conclusion, the large inter- and intrasubject variation in miR-122 and the recent recognition of its regulated release from the liver without hepatocyte death may complicate its interpretation in the clinic, but it is likely still valuable in certain contexts, such as in the setting of APAP-induced hepatotoxicity. Alternatively, MCSFR may be elevated to a greater degree in severe idiosyncratic DILI. GLDH was a sensitive biomarker for detection of DILI and should be useful in certain clinical contexts to exclude muscle injury as a source of serum biomarkers.[22] K18, FABP1, and ccK18 were also highly sensitive for DILI detection. OPN, K18, and MCSFR show promise as biomarkers that can identify those DILI patients who will succumb to a DILI event unless they receive a transplant. The combined use of K18, MCSFR, and MELD score improved specificity without reducing the sensitivity compared with use of a MELD score of $\geq$20 alone. Based on the data reported here, follow-up initiatives should include 1) further exploration of the prognostic value of the biomarkers endorsed by regulatory agencies through broad application in clinical trials with serial sample collection, 2) correlation of the mechanism of DILI with the performance of the biomarkers (e.g., intrinsic DILI versus immune activation), and 3) assessment of the performance of biomarkers in drug-induced versus other causes of liver injury (e.g., viral hepatitis or autoimmune hepatitis). These efforts will allow the most promising biomarkers to be validated and qualified for routine use in clinical DILI assessment.

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