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Serum Biomarkers Correlated with Liver Stiffness Assessed in a Multi-center Study of Pediatric Cholestatic Liver Disease

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Abstract

Background & Aims: Detailed investigation of the biological pathways leading to hepatic fibrosis and identification of liver fibrosis biomarkers may facilitate early interventions for pediatric cholestasis.

Approach & Results: A targeted ELISA-based panel of 9 biomarkers (lysyl oxidase (LOX), tissue inhibitor matrix metalloproteinase 1 (TIMP1), connective tissue growth factor (CTGF), interleukin-8 (IL-8), endoglin, periostin, mac-2-binding protein (mac2-BP), matrix metalloproteinase-3 and −7 (MMP-3, MMP-7) was examined in children with biliary atresia (BA, n=187), alpha-1 antitrypsin deficiency (A1AT, n=78) and Alagille syndrome (ALGS, n=65) and correlated with liver stiffness (LSM) and biochemical measures of liver disease. Median age and LSM were 9 years and 9.5 kPa. After adjusting for covariates, there were positive correlations between LSM and endoglin ($p=0.04$), IL-8 ($p<0.001$) and MMP-7 ($p<0.001$) in BA. The best prediction model for LSM in BA using clinical and lab measurements had an $R^2=0.437$; adding IL-8 and MMP7 improved R^2 to 0.523 and 0.526 (both $p<0.0001$). In A1AT, CTGF and LSM were negatively correlated ($p=0.004$); adding CTGF to a LSM prediction model improved R^2 from 0.524 to 0.577 ($p=0.0033$). Biomarkers did not correlate with LSM in ALGS. A significant number of biomarker/lab correlations were found in BA but not A1AT or ALGS.

Conclusions: Endoglin, IL-8, and MMP-7 significantly correlate with increased LSM in children with BA, while CTGF inversely correlates with LSM in A1AT; these biomarkers appear to enhance prediction of LSM beyond clinical tests. Future disease-specific investigations of change in these biomarkers over time and as predictors of clinical outcomes will be important.

Keywords

Alagille syndrome; alpha-1 antitrypsin deficiency; biliary atresia; cholestasis; cirrhosis; non-invasive; pediatric liver disease

Background

Detailed investigation of the biological pathways that lead to early and severe fibrosis and identification of non-invasive biomarkers of liver fibrosis can facilitate early interventions for pediatric cholestatic liver disorders. Biomarkers identified by genomic, proteomic, and metabolomic technologies offer new strategies to study etiopathogenesis, inform clinical decision-making or predict outcomes in pediatric liver diseases (1–4). However, each liver disease is unique with different contributory factors and the likely interplay of anatomy, epigenetics, fibrosis, inflammation, and vasculopathy.

Biliary atresia (BA), alpha-1 antitrypsin deficiency (A1AT) and Alagille syndrome (ALGS) represent an important group of congenital cholestatic disorders. BA is a fibro-obliterative disease of the biliary system that presents in the early neonatal period with aggressive progression to cirrhosis within the first year of life (5–9) and continues to be the leading indication for liver transplantation (LT) in children. Inherited liver disorders such as A1AT and ALGS, have a different natural history and distinct pathophysiology from BA and one another. Children with A1AT who are homozygous for the classic mutant Z allele have misfolding and defective secretion of the A1AT protein, with potential to progress to early cirrhosis with or without lung disease (10). However, it is unknown what factors influence the highly variable disease progression and risk of fibrosis seen in A1AT (11). ALGS, a developmental disorder manifest by bile duct paucity and cholestasis often with severe pruritus and extrahepatic involvement, including cardiac, vascular, and renal abnormalities (12) also has unpredictable progression of fibrosis, although the majority of patients are transplanted by 18 years of age for persistent cholestasis or complications of portal hypertension (13).

Single center studies in children with a variety of fibrotic liver diseases have identified specific markers reflecting matrix deposition, hepatic stellate cell activation, collagen turnover, and chemoattractant expression (2–4, 14) that correlate with histologic measures of fibrosis. Meanwhile, imaging modalities have advanced and may further augment our ability to monitor the progression and regression of liver fibrosis. Vibration-controlled transient elastography (TE, FibroScan®) is a non-invasive, painless alternative to liver biopsy that utilizes shear wave velocity to measure liver stiffness, an indirect measure of fibrosis. Use of TE to detect significant fibrosis in children has been validated by liver biopsy in single center studies of both hepatocellular and hepatobiliary diseases (15–21), however the availability of TE as a clinical tool in the pediatric setting is relatively expensive and as such currently limited. Identification of relevant commercially available serum biomarkers of fibrosis and their correlation with transient elastography may inform progression or severity of fibrosis where TE is unavailable and translate into novel markers/endpoints for clinical trials of evolving anti-fibrogenic therapies.

The FibroScan in Pediatric Cholestatic Liver Disease (FORCE) study is an ongoing, prospective, multi-center study (NCT02922751) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)/National Institutes of Health (NIH)-supported Childhood Liver Disease Research Network (ChiLDReN). This rich resource positioned the network to conduct investigations with the primary objective of identifying serum biomarkers associated with FibroScan™-based liver stiffness measurement (LSM) in children with BA, A1AT, and ALGS. The scientific rationale for the selection of the targeted nine biomarker panel is described in detail (Supplemental Document 1). These biomarkers potentially assess a diverse array of pathways and physiologic mechanisms that may interact, leading to fibrosis. Secondary objectives include investigating the association of these biomarkers with clinical characteristics, biomarker indices, and laboratory determinants of liver disease and ability to enhance prediction of LSM beyond standard clinical tests.

Methods

FORCE includes the collection of serum at the time of FibroScan™ in participants whose clinical status has been well characterized in three distinct prospective longitudinal databases (Prospective Database of Infants with Cholestasis [PROBE, NCT00061828], Biliary Atresia Study in Infants and Children [BASIC, NCT00345553], and Longitudinal Observational Study of Genetic Causes of Intrahepatic Cholestasis [LOGIC, NCT00571272]). This study focused on baseline evaluation of FORCE participants less than 21 years of age with BA, A1AT, or ALGS with their native liver who had a valid LSM and serum available for biomarker investigation.

Clinical data including medical history, physical examination, interval events, and laboratory values, along with biosamples and LSM were collected in FORCE and the parent longitudinal database. Serum samples were collected on the same day as LSM and immediately processed and stored at −80 C. Inclusion and exclusion criteria in FORCE as well as LSM requirements have been described previously in detail (22).

Briefly, BA participants all had a confirmed diagnosis of BA determined by chart review including review of pertinent diagnostic biopsy reports, or radiologic reports and were all post Kasai hepatoportoenterostomy with native liver. ALGS participants in this study met strict diagnostic criteria in which there was a combination of a family history of ALGS, the presence of paucity of interlobular bile ducts on liver biopsy, the identification of a JAGGED1 or NOTCH2 mutation, and clinical criteria (cardiac, ocular, vertebral, renal, facial, cholestasis) with evidence of clinical, biochemical or histological liver disease. Patients with A1AT were defined as having low serum A1AT concentrations (< lower limit of normal for laboratory) with PIZZ or PISZ phenotype or genotype for participants prior to liver transplantation with liver disease associated with A1AT. Children with known polysplenia or asplenia, situs inversus, clinically significant ascites, an implantable active medical device (such as a pacemaker or defibrillator), an open wound near the testing site, current pregnancy, or who had undergone liver transplantation were not eligible.

Liver stiffness (reported in kPa) was measured by vibration-controlled TE in nonfasted and nonsedated participants, using FibroScan according to the manufacturer’s instructions

(Echosens, Waltham, MA). All operators were trained and certified at each site by a designated trainer from Echosens to perform FibroScan™ measurements to ensure consistent and standardized acquisition of complete data. A valid LSM required at least ten valid measurements using the appropriate probe (S or M) and exam type (S1 or S2) based on thoracic perimeter and skin capsule distance with interquartile kPa range/median kPa value <30% (23).

LSM was categorized using previously described liver biopsy supported thresholds derived from children with cholestatic or chronic liver diseases and a meta-analysis derived reference range for normal liver stiffness in healthy children (20, 24–29). For analyses, the following categories were applied: <6 (no or mild fibrosis, F0/F1), 6 to <10 (significant fibrosis, ≥F2) 10 to <15 (bridging fibrosis, F3/4), and 15–75 (cirrhosis, F4) kPa. To address the potential impact of cardiac and venous congestion on LSM, for participants with ALGS only, severe cardiac disease was defined as having ≥1 of the following: pulmonary valve stenosis, tetralogy of Fallot, ventricular/atrial septal defect, pulmonary atresia, and/or aortic coarctation. All other cardiac defects were considered mild.

A PubMed search using the following terms: hepatic[All Fields] AND (“liver cirrhosis”[MeSH Terms] OR (“liver”[All Fields] AND “cirrhosis”[All Fields]) OR “liver cirrhosis”[All Fields] OR (“liver”[All Fields] AND “fibrosis”[All Fields]) OR “liver fibrosis”[All Fields]) AND (“biomarkers”[MeSH Terms] OR “biomarkers”[All Fields]) AND (“child”[MeSH Terms] OR “child”[All Fields] OR “children”[All Fields])) on November 1, 2018 yielded 30 peer reviewed articles providing the biological basis, scientific and clinical rationale for the targeted nine biomarker panel. Supplemental Document 1 summarizes what is known about each biomarker and its relationship to fibrosis or liver stiffness, in the context of pediatric liver disease when available.

ELISA-based measurement of these nine targeted biomarkers (lysyl oxidase (LOX, Mybiosource, Cat #MBS039099, San Diego, CA), tissue inhibitor matrix metalloproteinase 1 (TIMP1, R&D, Cat #DTM100, Minneapolis, MN), connective tissue growth factor (CTGF, Mybiosource, Cat # MBS266000, San Diego, CA), interleukin-8 (IL-8, Millipore, Cat # HCYTOMAG-60K-01, Burlington, MA), endoglin (Sigma-Aldrich, Cat # RAB0171, Burlington, MA), periostin (Abcam, Cat # AB213816, Cambridge, UK), mac-2-binding protein (mac2-BP, Mybiosource, Cat # MBS108990, San Diego, CA), matrix metalloproteinase-3 (MMP-3, R&D, Cat# DMP300, Minneapolis, MN) and matrix metalloproteinase-7 (MMP-7, R&D, Cat# DMP700, Minneapolis, MN) were performed according to the manufacturer’s instructions. Serum samples were run in duplicate and randomly aliquoted onto 96-well plates with six internal controls (frozen sera from healthy controls with normal liver biochemistries stored at −80 C). Measurements of each analyte were in the linear portion of the response curve for each biomarker.

Concentrations for selected biomarkers were displayed visually using boxplots. Scatter plots with LOESS (locally estimated scatterplot smoothing) lines were used to visually inspect associations between biomarker concentrations and clinical characteristics. Differences among disease groups were compared using Kruskal-Wallis tests for continuous variables and Chi-Square test for categorical variables. Biomarker differences in participants with

clinically evident portal hypertension status (CEPH, absent, possible, definite) using a previously described research definition (30) were also analyzed and tested with Kruskal-Wallis tests. Correlations with LSM were estimated using Spearman's rank correlation for biomarkers and other clinical parameters; multivariable linear regression was used to examine these associations controlling for age, total bilirubin (TB), albumin, GGT and AST. Residuals were used to evaluate regression model assumptions. Log transformations were applied to all biomarkers except TIMP-1 and MMP-7 to account for skewed distributions prior to regression modeling.

To evaluate whether the targeted biomarkers improve the ability to predict LSM in each diagnosis group beyond clinical characteristics and laboratory measures, a linear regression with forward step-wise selection (inclusion criteria; $p \leq 0.10$) was performed first to develop a parsimonious model including all clinical labs and features as candidate predictors for predicting LSM; the targeted biomarkers were then added one at a time to this model to evaluate which biomarker(s) further explain remaining variance in LSM and significantly improve the model fit. For all three disease groups, 50 multiply imputed data sets were utilized to complete any missing values. Rubin's rule was used to provide pooled results based on parameters obtained from each of the 50 data sets.

Serum samples from 100 children with similar distributions of age- and sex-matched children with normal liver biochemistries and without known liver disease served as case controls (Discovery Life Sciences, Los Osas, CA) for biomarker comparisons only. TE data was not available for case controls. Regression models adjusted for age, sex and race were performed to compare biomarkers levels in controls vs liver disease groups. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc, Cary, NC, USA).

Written informed consent was obtained from caregivers or the participant, and assent was obtained from the child when appropriate according to local Institutional Review Board (IRB) rules. This study was approved by local IRBs and complied with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Results

Participants

The FORCE study obtained valid baseline LSM exams on 458 participants enrolled from November 2016 to August 2019 (22). The current study included a subset of 330 (72%) FORCE participants (n=187 with BA, n=78 with A1AT, n=65 with ALGS) that had baseline serum samples available for biomarker ELISA analysis. Age was significantly greater in FORCE participants with available serum compared to those without (median 9.2 vs 6.1 years, $p<0.001$); sex, race, and ethnicity were not significantly different (Supplemental Table 1). Demographic characteristics of the study participants are shown in Supplemental Table 2. Ethnicity was not available for controls. BA participants had a higher percentage of females; ALGS participants were older; A1AT had the lowest percentage of Hispanic/Latino participants and highest percentage of White participants. Mean age at Kasai for 182 BA participants was 57 days (± 24).

Clinical and laboratory characteristics

Overall, median age was nine years, LSM 9.5 kPa, platelet count 216X10^3/ uL, TB 0.6 mg/dL, albumin 4.3 g/d, AST 57 U/L and GGT 78 U/L. Significant differences in all clinical variables except albumin were observed amongst the three disease groups (Table 1). Median LSM was highest (12.8 kPa) and platelet count was lowest (145 ×10^3/uL) in BA participants. A higher percentage of BA had splenomegaly (40%) compared to A1AT (4%) or ALGS (17%). A1AT had the lowest median LSM (6.3 kPa). Among ALGS, mean LSM between those with a severe cardiac disease (n=22) vs. mild cardiac disease (n=43) were not different (10.7 kPa vs. 11.5; median 9.2 vs. 9.0, data not shown). TB, GGTP, AST and ALT were highest in ALGS. Only ALGS demonstrated growth impairment, with median z-scores below zero in height, weight, and BMI.

Biomarker profiles

MMP-7 and endoglin levels were significantly higher in BA (both p<0.001) than controls (Table 2). Compared to controls, TIMP-1 concentrations were similar in ALGS but lower in both BA and A1AT (p<0.001). Among the disease groups, MMP-7 and endoglin levels were higher in BA than A1AT and ALGS (Figure 1). Among the nine biomarkers studied, the concentrations of all except TIMP-1, MMP-7 and endoglin were higher in age and sex-matched healthy controls with no known liver disease than in one or more of the three disease groups, BA, A1AT, or ALGS. (Table 2).

Biomarker correlations with participant age varied by liver disease group (Supplemental Document 2). In BA, MMP-7 had the strongest negative correlation with age (r=−0.38, p<0.001); in A1AT, TIMP-1 (r=−0.34, p=0.003) and CTGF (r=−0.37, p=0.001) most strongly correlated with age.

Biomarker and LSM correlations in BA

In BA, LSM correlated with MMP-3 (r =0.19, p=0.02), endoglin (r =0.27, p<0.001), periostin (r=0.22, p=0.01), IL-8 (r=0.47, p<0.001) and MMP-7 (r=0.45, p<0.001) (Figure 2). After adjusting for covariates (age, TB, albumin, GGTP, and AST), the positive association between LSM and IL-8 (p<0.001), MMP-7 (p<0.001), and endoglin (p=0.02) remained significant. Periostin (p=0.051) and MMP-3 (p=0.21) were not significant after adjusting for other covariates. The best parsimonious prediction model for LSM in BA based on clinical characteristics and laboratory measurements (spleen size, PELD, platelets, AST) had an R^2=0.437. Adding IL8 and MMP7 further improved the R^2 to 0.523 (<0.0001) and 0.526 (p<0.0001), respectively (Table 3). IL-8 and MMP-7 concentrations were higher in BA participants with higher LSM (Figure 3). Endoglin concentrations were also higher in in BA with higher LSM. CTGF had no clear relationship with LSM in BA.

BA had the largest sample size and highest number of significant biomarker/biomarker correlations (10 pairs) and significant biomarker correlations with LSM (Figure 2). Specifically, in BA, lysyl oxidase (LOX) and Mac-2BP were correlated with each other (r=0.55, p<0.001), and IL-8 was strongly correlated with MMP-7 (r=0.46, p<0.001), endoglin (r=0.48, p<0.001) and MMP-3 (r=0.41, p<0.001).

Biomarker and LSM correlations in A1AT

Only CTGF was correlated with LSM in A1AT (R = −0.38, p=0.01) (Supplemental Figure 1); CTGF was lower in A1AT participants with higher LSM (Figure 3). The negative association between LSM and CTGF remained significant (p=0.004) after adjusting for covariates. The best parsimonious prediction model for LSM based on clinical and lab measurements (APRI, total bilirubin, spleen size) had an R^2=0.524. Adding CTGF (log transformed) improved R^2 to 0.577 (p=0.0033) (Supplemental Table 3).

While correlations were not significant, MMP-7 and endoglin concentrations trended higher in A1AT participants with higher categories of LSM (Figure 3). Among A1AT, LOX was correlated with Mac-2BP (r=0.51, p=0.001, Supplemental Figure 1).

Biomarker and LSM correlations in ALGS

No biomarkers were significantly correlated with LSM in ALGS (Figure 3 and Supplemental Figure 2). However, among ALGS, LOX and Mac-2BP were correlated (r=0.48, p<0.05) with one another; similarly, IL-8 and endoglin were strongly correlated with each other (r=0.45, p<0.01), as were MMP-7 and TIMP1 (r=0.52, p<0.001) (Supplemental Figure 2).

Biomarker correlations with clinical features and laboratory measurements in BA

BA had the most significant biomarker/laboratory (40 pairs) correlations. Among BA, endoglin, TIMP-1, periostin, IL-8, and MMP-7 were associated with almost all clinical and laboratory markers of liver disease progression (Figure 4). Endoglin, TIMP1, IL-8 and MMP-7 were all significantly and negatively correlated with age (r= −0.22 to −0.38, p<0.01 for all) and albumin (r= −0.18 to −0.29, p<0.05 or less for all). GGTP correlated with TIMP-1, IL-8, and MMP-7 (r=0.38–0.41, p<0.001), as did AST (r= 0.28–0.53, p<0.001 for all). In BA, platelets had the strongest correlation with TIMP-1, while AST to platelet ratio index (APRI) had the strongest correlation with IL-8. Among BA, endoglin (p=0.003) and IL-8 (p=0.02) were highest in participants with definite clinically evident portal hypertension (CEPH) vs absent or possible CEPH (Supplemental Table 4). TIMP-1 was lowest in BA participants with definite CEPH (p<0.001) vs absent or possible CEPH; LOX and Mac-2BPGi were also lowest in BA with definite CEPH (p=0.04, p=0.008, respectively). MMP-7 was highest (7.8 ng/mL) in BA with definite CEPH vs possible (6.0 ng/mL) and absent (5.2 ng/mL) CEPH but not significant (p=0.19)

Biomarker correlations with clinical features and laboratory measurements in A1AT

A1AT had the least statistically significant biomarker/laboratory (7 pairs) correlations (Supplemental Figure 3). Periostin was not associated with any laboratory associated with liver injury. Among A1AT, TIMP-1 and CTGF were negatively associated with age. Platelet count was correlated with TIMP-1 and CTGF. AST correlated the highest with IL-8 (r=0.36) and MMP-7 (r=0.32, both p<0.05). APRI had the strongest correlation with IL-8 (r=0.42, p<0.05). Among A1AT, CTGF was the lowest in participants with definite CEPH (288.6 pg/mL) vs absent CEPH (572.5 ng/mL, p=0.05), consistent with its correlation with LSM (Supplemental Table 4). Only 5 patients with A1AT had definite CEPH. Similar to BA,

TIMP-1 was lowest among A1AT with definite CEPH (108.7 ng/mL, p=0.04) vs possible (114 ng/mL) and absent (136.6 ng/mL).

Biomarker correlations with clinical features and laboratory measurements in ALGS

ALGS also had few statistically significant biomarker/laboratory (9 pairs) correlations (Supplemental Figure 4). Among ALGS, TIMP-1 was correlated with bilirubin, AST, APRI, and PELD (r=0.41–0.54, all p<0.01). IL-8 also correlated with these same factors (r=0.44–0.50, all p<0.01).

Discussion

Discovery and validation of disease-specific serum and imaging biomarkers of liver fibrosis may inform the unique etiopathogenesis of BA, A1AT, and ALGS and identify distinct targets as well as clinical endpoints for future anti-fibrotic therapies. The mechanisms of liver disease are numerous and include genetic etiologies impacting development or protein trafficking, inflammation, biliary obstruction, vascular abnormalities and congestion, cell death, and fibrogenesis; all could impact liver stiffness. Overall, the study cohort was quite young (median <10 years); yet median LSM was 9.5 kPa, consistent with significant fibrosis, despite preserved liver function with only mildly elevated biochemistries. TE capability is not ubiquitous among most pediatric hospitals, however commercially available serum biomarkers validated by TE may offer additional non-invasive alternatives to assess or predict worsening liver stiffness or disease progression.

Biomarker correlation with LSM and clinical parameters of liver disease varied widely among BA, A1AT, and ALGS highlighting potential disease-specificity of serum and imaging biomarkers of liver injury. BA is biologically distinct and characterized by extrahepatic biliary obstruction with progression to cirrhosis much earlier and more frequently than A1AT and ALGS. As such, it was not surprising that BA participants post Kasai had the highest median LSM (12.8 kPa), lowest platelet count, and highest incidence of splenomegaly despite not having the highest TB, GGT, AST, or ALT (ALGS had the highest levels). In a prior study of 30 infants at the time of Kasai, a LSM of 15.1 kPa or higher predicted cirrhosis (Metavir ≥ F4 by liver biopsy), however this cohort was much younger (mean 76 days) than our study cohort (27). Interestingly, mean MMP-7 levels were also two-fold higher in BA compared to controls, A1AT, or ALGS. This is consistent with other studies showing that serum MMP-7 is significantly higher in BA while also offering high diagnostic accuracy in distinguishing BA from other cholestatic liver disorders (31–33) and correlation with liver fibrosis (34). Notably, in our study, BA had highest MMP-7 concentrations in the first year of life and decreased to control concentration levels by early adulthood. Among BA, MMP-7 was also highly correlated with GGT, AST, and LSM. The addition of MMP-7 improved clinical and lab-based model prediction of LSM in BA by explaining an additional 9% of the variation in LSM. Based on these important biomarker and liver stiffness associations, MMP-7 appears to be a reliable biomarker of worsening fibrosis in BA. Matrix metalloproteinases (MMP) have been found to be involved in the activation of hepatic stellate cells and increased extracellular matrix, both of which are associated with the fibrogenic mechanisms of BA (35, 36). Notably, in a large public domain

single cell human RNA data set (37), MMP-7 was highly expressed in cholangiocytes and enriched in individuals with cirrhosis. MMP-7 also appears to be the most consistently expressed marker in animal models of BA (38–40).

In BA, endoglin and IL-8 were positively correlated with LSM; in addition to MMP-7, these biomarkers correlated with at least seven of the 10 clinical parameters of interest. These three biomarkers are involved in hepatic stellate cell differentiation (36), biliary epithelial tissue remodeling (41), and chemoattraction in cholangiocytes (42, 43), respectively, and represent pathways leading to increased liver stiffness and potential targets for anti-fibrotic therapies. Interestingly, endoglin, a glycoprotein co-receptor for TGF-β involved in cytoskeletal organization was the only biomarker that was universally higher in all liver disease groups vs controls, though only significantly higher in BA, likely due to its larger sample size. Our reported mean endoglin level of 11.1 ng/mL in BA post-Kasai is higher than previously reported in a younger post-Kasai cohort (7.8 ng/mL) (44).

The negative correlation between LSM and CTGF among A1AT is intriguing and paradoxical in the context of anticipated liver fibrosis in A1AT. Interestingly, CTGF also had a significant negative correlation with age and portal hypertension severity. CTGF is tightly regulated by HNF4alpha and YAP. HNF4alpha is a transcription factor known for its roles in maintaining balance between hepatocyte differentiation versus quiescence and organization of the sinusoidal endothelium during development (45, 46). YAP, a downstream effector of the Hippo pathway, is known to promote liver regeneration (47, 48) in response to injury or parenchymal loss. It is possible that serum CTGF levels may reflect the real-time state of the hepatocyte, in this case, quiescence, in A1AT. Low CTGF may reflect a defective compensatory hepatocyte proliferation in response to hepatocyte injury in A1AT despite increased fibrosis or liver stiffness. Of note, CTGF is also positively correlated with platelet count in A1AT and seems to be a consistent biomarker of worsening liver disease or emerging portal hypertension in A1AT. The addition of CTGF also improved clinical and lab-based prediction model of LSM in A1AT.

With the exception of endoglin (highest in BA), TIMP-1 (highest in ALGS), and MMP-7 (highest in BA), biomarker concentrations of our targeted panel were surprisingly higher in age-matched healthy controls compared to those with liver disease. Given what is known about the putative role of these biomarkers in liver fibrosis, this was an unexpected but important finding not previously described. This suggests that levels of some collagen/matrix markers may be confounded by factors inherent to liver disease such as cholestasis or suboptimal nutrition. For example, periostin levels were nearly two-fold higher in controls than any of the liver disease groups. Children with cholestatic liver disease commonly have growth stunting, manifest vitamin D deficiency due to poor absorption of fat soluble vitamins, and have osteopenia or fractures (49, 50). Hence, it is critical to acknowledge that some collagen based biomarkers may be more challenging to interpret in the context of specific pediatric liver disorders and age groups. Alternatively, it is possible that some of these collagen/matrix markers may have less biological relevance in pediatric liver disease than previously thought.

This study has some limitations. Although the panel was selected based on a rigorous review of peer-reviewed literature supporting their putative value as liver fibrosis biomarkers, only nine targeted biomarkers were studied. Changes in biomarker serum levels may reflect altered clearance rather than decreased or increased production; they may also not directly reflect changes within the liver. LSMs were not validated by liver biopsy in this study. Ideally, immunostaining of liver tissues to assess the intensity and distribution of biomarker expression can inform hypotheses about reasons for changes in biomarker levels in serum; however, liver biopsy was not considered feasible in the FORCE study. Our cross-sectional study design also does not indicate causation or allow us to measure the biomarkers at multiple time points to correlate with fibrosis progression as measured by Fibroscan and lab parameters. Mean serum concentration of MMP-7 levels among our much older BA cohort in this study (median age of 8.8 years) are lower than other studies reporting median MMP-7 levels at the time of BA diagnosis (median age 54–59 days) (33, 34). Post-Kasai status, age, duration of liver disease, and brand of ELISA kit are likely important factors. Lastly, there were fewer ALGS and A1AT participants than BA, resulting in a limited number of ALGS and A1AT participants with high LSM. This likely increased the risk of a Type II error for detecting significant associations between biomarkers and disease severity.

The correlations between biomarkers may shed light on mechanistic pathways that contribute to increased liver stiffness in specific pediatric liver disorders. Correlation of IL-8 and endoglin was demonstrated in both BA and ALGS participants. In BA, IL-8 also independently correlated with LSM and improved model prediction of LSM among BA. IL-8 is a chemokine involved in angiogenesis and liver inflammation while endoglin is overexpressed after liver or arterial injury. It is plausible that vascular abnormalities, common in BA (e.g., aberrant portal vein or obliterative venopathy) (51) and ALGS (e.g., cerebrovascular and renovascular malformations) (52, 53), may play a role in liver fibrosis or liver stiffness. While the correlation of IL-8 with LSM among ALGS was not significant ($p=0.09$) in a multivariable model, a larger disease population may have better elucidated this correlation.

Conclusion

Biomarker correlation with LSM and other clinical parameters of liver disease varied by type of pediatric cholestatic liver disease. This study provides evidence that endoglin, IL-8, and MMP-7 significantly correlate with increased LSM in children with BA, while CTGF inversely correlates with LSM in A1AT; these biomarkers also appear to enhance clinical characteristic and laboratory- based prediction of LSM and represent distinct but related mechanistic pathways of liver fibrosis and potential targets for antifibrotic therapies. As LSM via transient elastography is not yet clinically available or a standard of care in most pediatric hospitals, these commercially available biomarkers may offer additional non-invasive tools to guide decision making. However, disease type and age appear to be important factors in biomarker and LSM interpretation. Future investigations of imaging and serum biomarkers in pediatric cholestasis should use disease specific thresholds and would benefit from evaluation of change in biomarker levels over time as predictors of clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A1AT	alpha-1 antitrypsin deficiency
ALGS	Alagille syndrome
APRI	AST to platelet ratio index
AST	aspartate aminotransferase
BA	biliary atresia
BASIC	Biliary Atresia Study in Infants and Children
CEPH	clinically evident portal hypertension
ChiLDReN	Childhood Liver Disease Research Network
CI	confidence intervals
CTGF	connective tissue growth factor
FORCE	FibroScan⁻ in Pediatric Cholestatic Liver Disease
GPR	gamma glutamyl transpeptidase to platelet ratio
GGT	gamma glutamyl transpeptidase
INR	International Normalized Ratio
IQR	Interquartile Range
IRB	Institutional Review Board
LOESS	locally estimated scatterplot smoothing

LOGIC	Longitudinal Observational Study of Genetic Causes of Intrahepatic Cholestasis
LOX	Lysyl Oxidase
LSM	liver stiffness measurement
LT	liver transplant
Mac2-BP	mac-2-binding protein
MMP	Matrix metalloproteinases
NIH	National Institutes of Health
PELD	Pediatric End-Stage Liver Disease
PROBE	Prospective Database of Infants with Cholestasis
TB	total bilirubin
TE	transient elastography
TIMP1	tissue inhibitor matrix metalloproteinase 1

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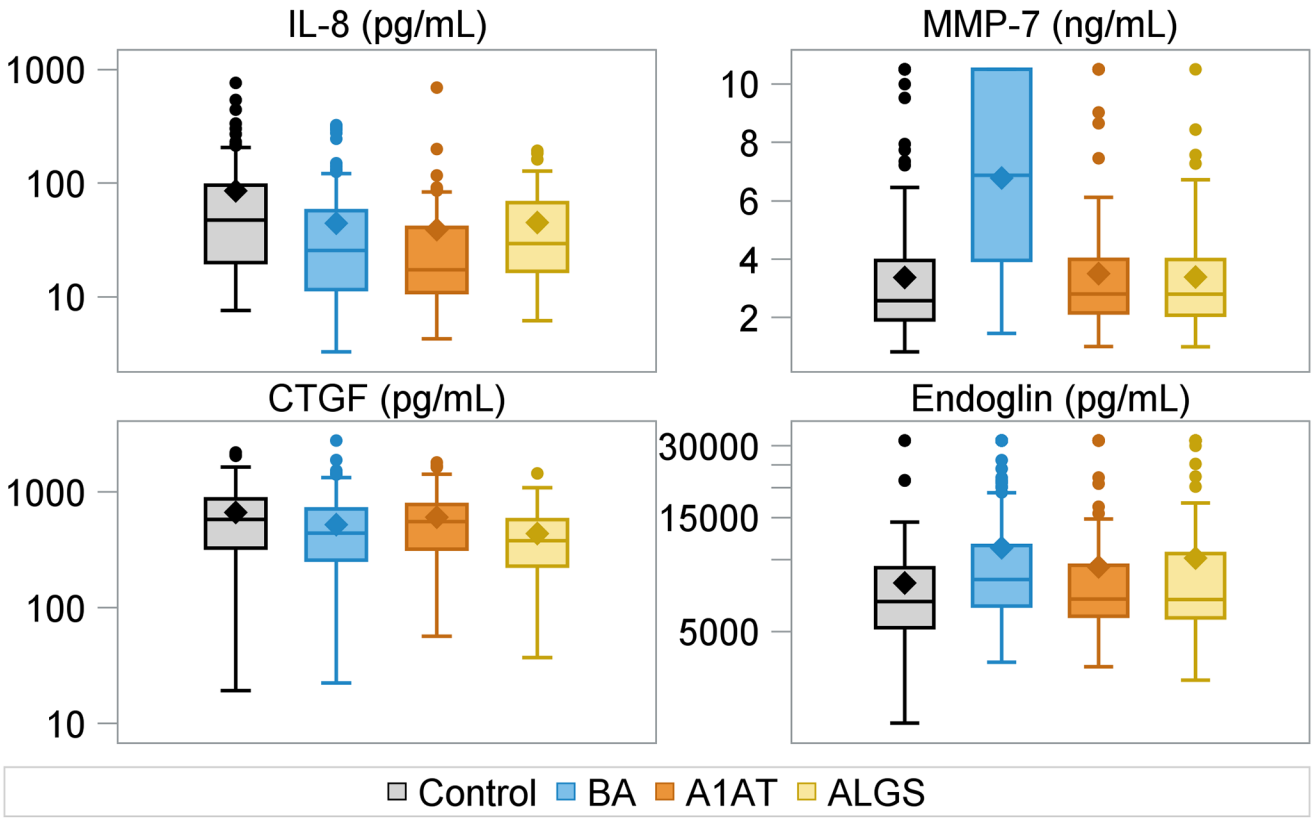

Figure 1.
Boxplots of selected biomarker concentrations (IL-8, MMP-7, CTGF, Endoglin) by diagnosis (control, BA, A1AT, ALGS).

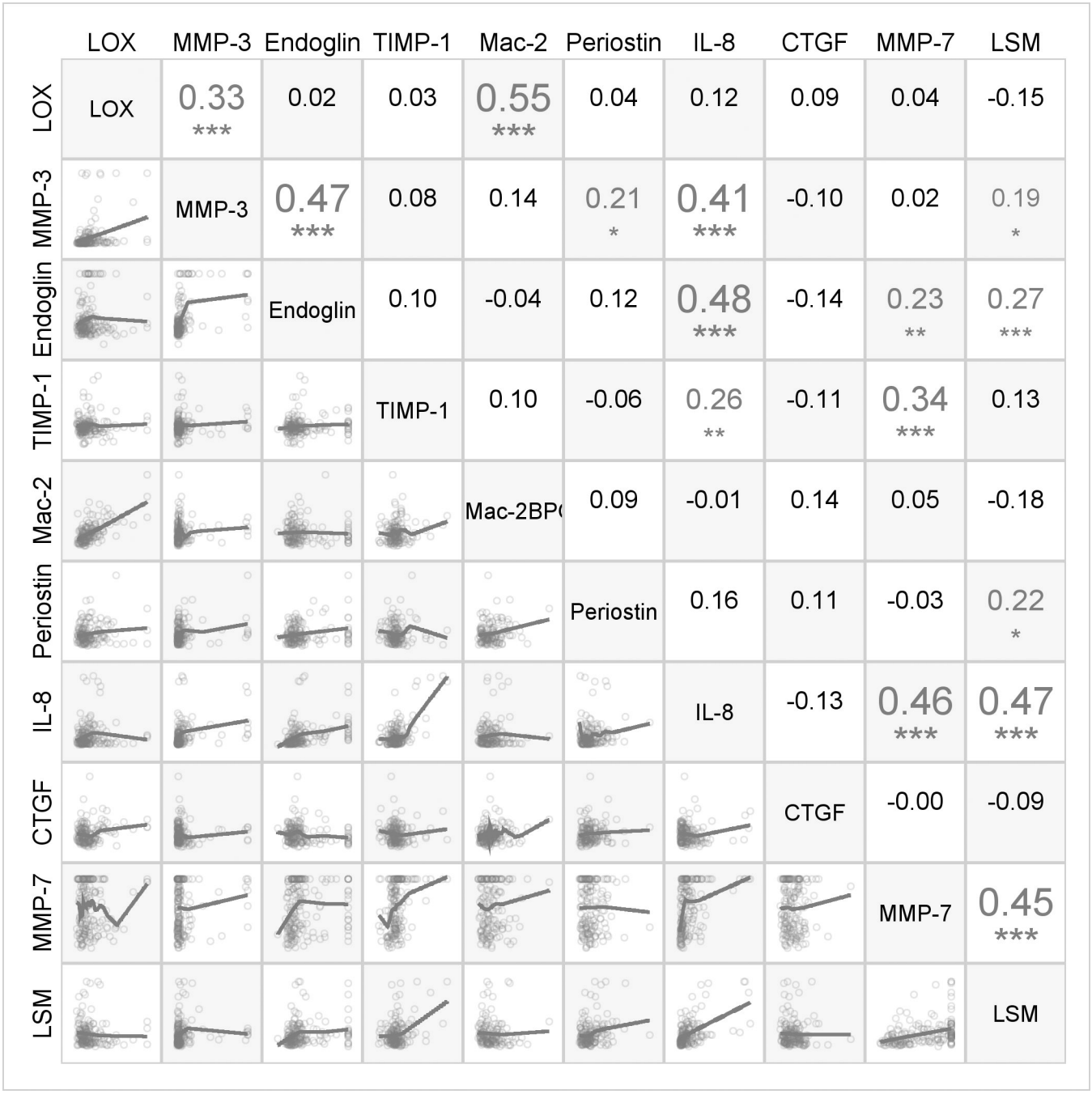

Figure 2.

Spearman correlations and scatter plots among biomarker concentrations and liver stiffness measurements for participants with BA. The numbers in the upper triangle of the grid display the Spearman correlation coefficients for pairs of variables. The size of the font corresponds to the magnitude of the correlation and the symbols below the numbers indicate significance levels after using multiple comparisons correction for false discovery rate: * $p<0.05$; ** $p<0.01$;*** $p<0.001$

The lower triangle of the grid displays scatter plots with LOESS lines.

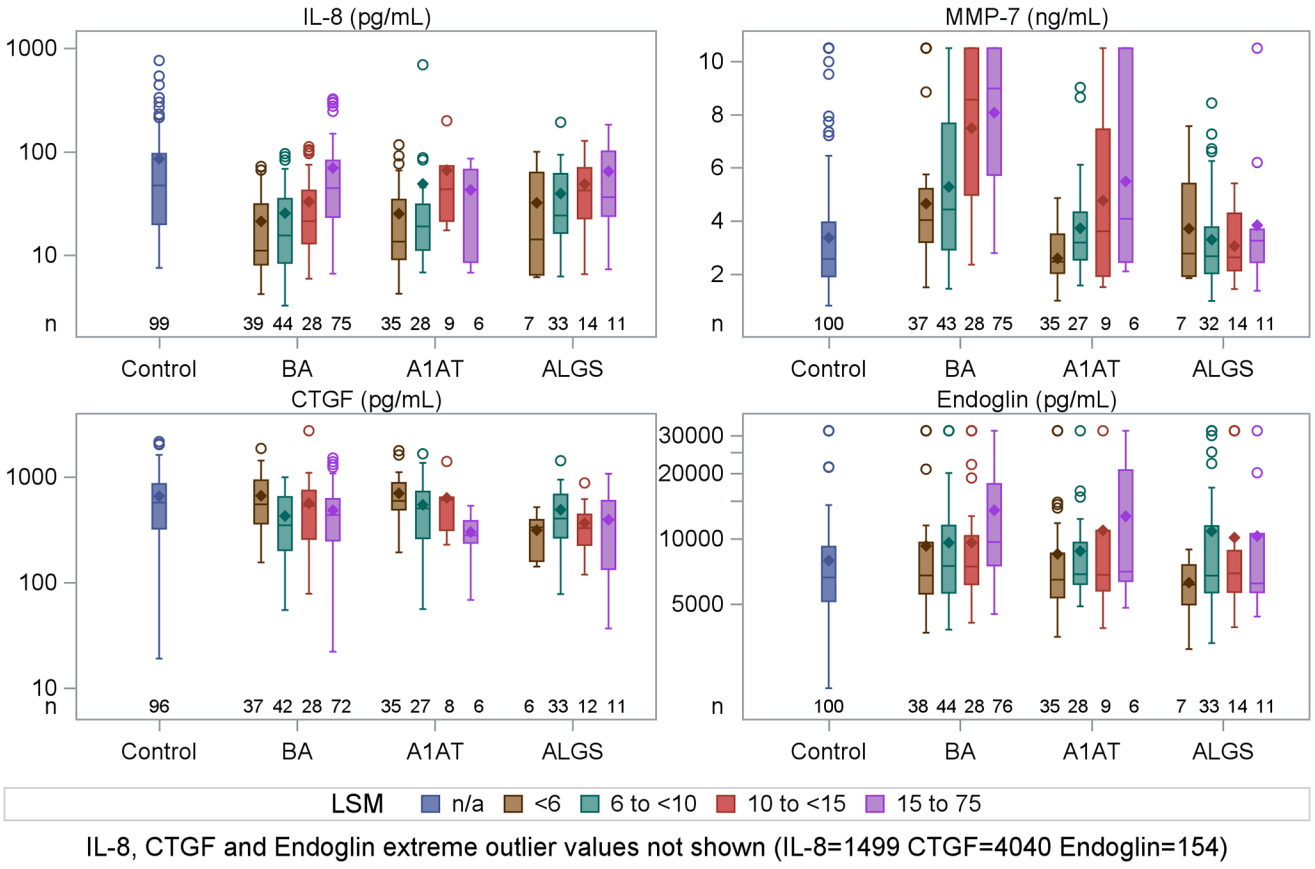

Figure 3.

Boxplots for selected biomarkers (IL-8, MMP-7, CTGF, Endoglin) by diagnosis (control, BA, A1AT, ALGS) and liver stiffness measurement category.

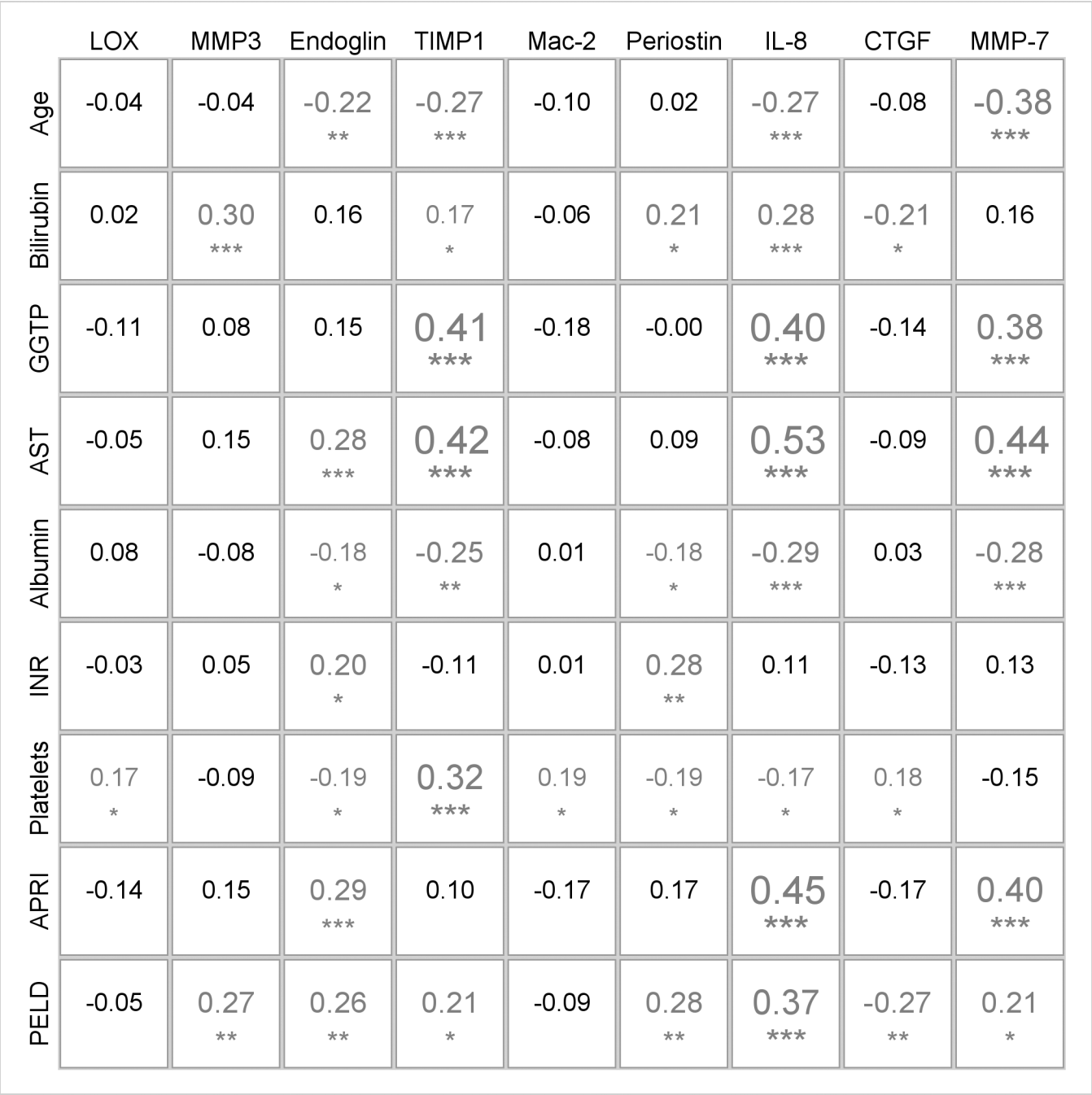

Figure 4.

Spearman correlations between biomarker concentrations and laboratory measurements for BA participants. The size of the font corresponds to the magnitude of the correlation and the symbols below the numbers indicate significance levels after using multiple comparisons correction for false discovery rate: * $p<0.05$;**$p<0.01$; ***$p<0.001$

Table 1.

Clinical characteristics of study participants by diagnosis

Characteristic	BA (n=187)	A1AT (n=78)	ALGS (n=65)	p-value*
FibroScan LSM (kPa)				
n	187	78	65	
Median (Q1, Q3)	12.8 (7.3, 20.0)	6.3 (5.1, 8.5)	9.0 (6.9, 12.5)	<0.001
Height Z-score				
n	186	76	65	
Median (Q1, Q3)	0.3 (−0.6, 0.7)	0.3 (−0.3, 1.0)	−1.2 (−2.0, −0.6)	<0.001
Weight Z-score				
n	186	76	65	
Median (Q1, Q3)	0.4 (−0.3, 1.0)	0.5 (−0.1, 0.9)	−1.1 (−2.1, −0.5)	<0.001
BMI Z-score				
n	186	76	65	
Median (Q1, Q3)	0.5 (−0.1, 1.1)	0.3 (−0.3, 0.9)	−0.5 (−1.3, 0.2)	<0.001
Total bilirubin (mg/dl)				
n	177	78	64	
Median (Q1, Q3)	0.6 (0.4, 1.1)	0.4 (0.2, 0.6)	1.1 (0.6, 2.9)	<0.001
GGTP				
n	164	72	56	
Median (Q1, Q3)	79.5 (29.5, 175.5)	26.0 (17.0, 50.0)	352.5 (185.0, 797.0)	<0.001
AST				
n	177	78	64	
Median (Q1, Q3)	54 (34, 94)	46 (31, 60)	110 (74, 178)	<0.001
ALT				
n	178	78	64	
Median (Q1, Q3)	54 (35, 94)	53 (35, 77)	140 (88, 243)	<0.001
Albumin (g/dl)				
n	176	76	63	
Median (Q1, Q3)	4.3 (3.9, 4.5)	4.4 (4.2, 4.6)	4.4 (3.9, 4.5)	0.08
n (%) <3.0	3 (2%)	1 (1%)	2 (3%)	0.70
INR				
n	148	60	56	
Median (Q1, Q3)	1.1 (1.0, 1.2)	1.1 (1.0, 1.1)	1.0 (1.0, 1.1)	<0.001
Spleen size (cm below costal margin)				
n	187	78	65	
Median (Q1, Q3)	1 (0, 5)	0 (0, 0)	0 (0, 0)	<0.001
n (%) >2 cm	75 (40%)	3 (4%)	11 (17%)	<0.001
Platelet count				

Characteristic	BA (n=187)	A1AT (n=78)	ALGS (n=65)	p-value*
n	183	75	62	
Median (Q1, Q3)	145 (84, 242)	269 (220, 332)	251 (198, 325)	<0.001
n (%) <150	92 (50%)	7 (9%)	9 (15%)	<0.001
APRI				
n	176	75	62	
Median (Q1, Q3)	0.9 (0.4, 2.4)	0.4 (0.3, 0.6)	1.2 (0.6, 2.1)	<0.001
n (%) <1.5	106 (60%)	66 (88%)	39 (63%)	<0.001
PELD				
n	141	58	55	
Median (Q1, Q3)	−10.3 (−12.7, −5.6)	−13.2 (−16.0, −10.7)	−6.3 (−10.1, 0.8)	<0.001

*Kruskal-Wallis test for continuous variables. Chi-Square tests for binary variables.

Table 2.

Biomarker Comparisons* between disease groups and controls

		Control	BA	A1AT	ALGS
LOX (ng/mL)	Mean	5.0	3.9	3.8	**3.1**
	p-value	Ref.	0.045	0.22	**0.004**
MMP-3 (ng/mL)	Mean	12.1	**9.9**	**7.2**	9.3
	p-value	Ref.	**0.003**	**<0.001**	0.4
Endoglin (pg/mL)	Mean	7,958	**11,117**	9,295	10,118
	p-value	Ref.	**<0.001**	0.2	0.23
TIMP-1 (ng/mL)	Mean	171	**134**	**135**	176
	p-value	Ref.	**<0.001**	**<0.001**	0.29
Mac-2BPGi (ng/mL)	Mean	5.5	**3.9**	**4.0**	**3.5**
	p-value	Ref.	**0.002**	**0.003**	**<0.001**
Periostin (pg/mL)	Mean	6,968	**3,002**	**2,785**	**3,895**
	p-value	Ref.	**<0.001**	**<0.001**	**<0.001**
IL-8 (pg/ML)	Mean	99	**44**	**39**	**45**
	p-value	Ref.	**<0.001**	**<0.001**	**0.006**
CTGF (Pg/mL)	Mean	695	518	601	**434**
	p-value	Ref.	0.01	0.6	**0.001**
MMP-7 (ng/mL)	Mean	3.4	**6.8**	3.5	3.4
	p-value	Ref.	**<0.001**	0.26	0.41

* p-values based on Kruskal-Wallis tests

Table 3.

Linear regression models predicting LSM in BA (n=187) with biomarkers added individually to conventional laboratory measurements and clinical features

Best parsimonious model using conventional labs and clinical factors R^2= 0.473 (95% CI: 0.361, 0.574); Adj-R^2=0.461(0.349, 0.564)		
Term	**Estimate**	**p-value**
Spleen Size	0.0108	0.06
PELD	0.0165	<0.001
Platelets	−0.00079	<0.001
AST	0.0014	<0.001

Separate linear regression models predicting LSM with best parsimonious model based on conventional labs + target biomarker one at a time			
Term	**R^2 (95% CI)**	**Adj-R^2 (95% CI)**	**p-value**
Log_{10}(LOX)	0.478 (0.367, 0.579)	0.463 (0.351, 0.566)	0.19
Log_{10}(MMP3)	0.474 (0.363, 0.575)	0.459 (0.347, 0.562)	0.55
Log_{10}(Endoglin)	0.474 (0.363, 0.576)	0.460 (0.348, 0.563)	0.48
TIMP1	0.505 (0.397, 0.602)	0.491 (0.383, 0.590)	<0.001
Log_{10}(Mac2)	0.477 (0.366, 0.578)	0.462 (0.351, 0.565)	0.25
Log_{10}(Periostin)	0.480 (0.369, 0.581)	0.465 (0.353, 0.568)	0.15
Log_{10}(IL8)	0.523 (0.416, 0.617)	0.509 (0.402, 0.606)	<0.001
Log_{10}(CTGF)	0.475 (0.364, 0.577)	0.461 (0.349, 0.564)	0.35
MMP7	0.526 (0.419, 0.621)	0.513 (0.404, 0.610)	<0.001