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Protein biomarkers GDF15 and FGF21 to differentiate mitochondrial hepatopathies from other pediatric liver diseases

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Abstract

Background: Mitochondrial hepatopathies (MHs) are primary mitochondrial genetic disorders that can present as childhood liver disease. No recognized biomarkers discriminate MH from other childhood liver diseases. The protein biomarkers growth differentiation factor 15 (GDF15) and fibroblast growth factor 21 (FGF21) differentiate mitochondrial myopathies from other myopathies. We evaluated these biomarkers to determine if they discriminate MH from other liver diseases in children.

Methods: Serum biomarkers were measured in 36 children with MH (17 had a genetic diagnosis); 38 each with biliary atresia, α1-antitrypsin deficiency, and Alagille syndrome; 20 with NASH; and 186 controls.

Results: GDF15 levels compared to controls were mildly elevated in patients with α1-antitrypsin deficiency, Alagille syndrome, and biliary atresia-young subgroup, but markedly elevated in MH ($p < 0.001$). FGF21 levels were mildly elevated in NASH and markedly elevated in MH ($p < 0.001$). Both biomarkers were higher in patients with MH with a known genetic cause but were similar in acute and chronic presentations. Both markers had a strong performance to identify MH with a molecular diagnosis with the AUC for GDF15 0.93 ± 0.04 and for FGF21 0.90 ± 0.06. Simultaneous elevation of both markers $>$ 98th percentile of controls identified genetically confirmed MH with a sensitivity of 88% and specificity of 96%. In MH, independent predictors of survival without requiring liver transplantation were international normalized ratio and either GDF15 or FGF21 levels, with levels <2000 ng/L predicting survival without liver transplantation ($p < 0.01$).

Conclusions: GDF15 and FGF21 are significantly higher in children with MH compared to other childhood liver diseases and controls and, when combined, were predictive of MH and had prognostic implications.

INTRODUCTION

Pathogenic variants in genes encoding proteins required for mitochondrial bioenergetics functions, such as the oxidative phosphorylation system, constitute the primary mitochondrial disorders (MDs). There are over 300 recognized genetic causes involving both mitochondrial deoxyribonucleic acid (mtDNA) and nuclear DNA.[1,2] The clinical presentations are varied, often including prominent multisystem involvement with

Abbreviations: ChiLDReN, Childhood Liver Disease Research Network; EFS, event-free survival; FGF21, fibroblast growth differentiation factor 21; GDF15, growth differentiation factor 15; INR, international normalized ratio; IQR, interquartile range; IRB, institutional review board; MD, mitochondrial disorders; MH, mitochondrial hepatopathy; mtDNA, mitochondrial deoxyribonucleic acid; ROC, receiver operating curve.

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neuromuscular symptoms,[3] and categorized in recognizable clinical syndromic presentations.[4] MDs can affect the liver in up to 20% of patients. Mitochondrial hepatopathies (MHs) may present as acute liver failure, acute or chronic liver disease, fatty liver disease, cholestasis, or cirrhosis and may be difficult to distinguish from other etiologies.[4–7] At least 10 genetic causes of MH have been identified.[7] Many patients with MH have disorders of mtDNA maintenance caused by biallelic pathogenic variants in nuclear genes and leading to hepatic mtDNA depletion syndrome, or deletions of mtDNA. Currently, approximately half of patients clinically diagnosed as MH have an identifiable genetic cause.[7]

The recognition of MH is hampered by the lack of well-identified biomarkers that recognize MH within the large heterogenous group of childhood liver diseases. Lactic acidosis is common in liver dysfunction, particularly during liver failure, and neither lactate nor the lactate/pyruvate ratio differentiate MH from other causes of pediatric acute liver failure.[8] Current diagnosis relies on extensive genetic testing involving both nuclear and mtDNA, or on invasive testing by enzymatic assays of liver biopsies.[9] Thus, there is an unmet need for the discovery and validation of biomarkers that assist in selecting patients for this more extensive testing.

Fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) are metabolic homeostasis regulator proteins with paracrine and endocrine effects.[10–12] FGF21 is primarily secreted from the liver, but also adipocytes, myocytes, and pancreas, and increased secretion in MD is present in muscle as a myokine. GDF15 is mainly secreted from the kidney, liver, lung, pancreas, placenta, and prostate.[13] Expression of both is increased as part of the early integrated mitochondrial stress response.[14–16] The integrated mitochondrial stress response consists of the activation of a series of transcription pathways activated by mitochondrial dysfunction such as redox imbalance or mitochondrial membrane polarization defects.[14,17] Several studies within the context of neuromuscular disorders showed both proteins elevated compared to controls in patients with primary MD, and each markedly outperformed classic biomarkers of mitochondrial disease, such as lactate and pyruvate, for discrimination in receiver operating curve (ROC) analysis.[18–32] Both biomarkers are most elevated in patients with mtDNA maintenance defects (eg, *POLG*, MNGIE) and mitochondrial translation disorders (eg, *TMRU*), the categories most often associated with genetic causes of MH. GDF15 was more frequently elevated in neuromuscular MD than FGF21 when compared to controls, and in some studies, GDF15 had better sensitivity, and other ROC statistics compared to FGF21.[27–30] In other studies that used more comparison groups with other conditions, FGF21 and GDF15 had similar sensitivity but FGF21 had greater specificity.[24,33] In the broader presentation of multisystem disorders, some studies have shown good results for FGF21 in pediatric MD, whereas others indicated poor specificity, thus illustrating that the clinical context is very important in the evaluation of the clinical usefulness of these biomarkers.[33,34] No studies have thus far evaluated these biomarkers to identify MH in pediatric patients with liver disease. Past studies in adults showed elevated levels of GDF15 in certain adult cholestatic conditions and of FGF21 levels in NASH, illustrating the need for careful evaluation.[24,35,36]

In this study, we evaluated the performance of these protein biomarkers to distinguish MH from other childhood liver disorders. After the development of a pediatric reference range, we compared samples from patients with MH with samples from patients with several other pediatric liver disorders in a cross-sectional study. We further evaluated if these biomarkers could be used to assist in predicting the clinical outcome of MH.

METHODS

Patients

This cross-sectional study evaluated serum levels of FGF21 and GDF15 in patients from the Childhood Liver Disease Research Network (ChiLDReN) consortium, an NIDDK-NIH–funded multicenter consortium studying rare liver diseases in children including MH.[7] Participants or their parents provided informed consent for this study according to ChiLDReN protocols, which included the use of biobanked serum samples, under either a central institutional review board (IRB) at Salus or on an institution-specific IRB-approved protocol. Studies in Colorado were carried out under the related IRB-approved protocol (COMIRB 07-0736). Samples from patients with NASH were obtained with informed consent from biobanked serum samples (COMIRB 12-0069).

A control range was constructed from pediatric control samples under an IRB-approved study (COMIRB 20-2032). Control serum samples were obtained as remnant samples from the clinical laboratory at Children's Hospital Colorado, Aurora, Colorado. An additional 27 control serum samples were obtained from fasting patients presenting for upper gastrointestinal endoscopy, indicated for noninflammatory disease and without evidence of liver disease, for whom informed consent was obtained on an IRB-approved study (COMIRB 12-0069). The electronic health record for all control samples that had GDF15 or FGF21 results above the 90th percentile and all samples from patients age <6 months (mo) were reviewed for the presence of other clinical conditions that were considered significant and resulted in removal of these samples from the normal control group, including renal disease, ongoing liver disease, prematurity, and

hypoxic-ischemic injury. The ChiLDReN biorepository provided serum samples stored at −80°C, from children with MH, and available samples, enrolled in the MITO-HEP study (NCT01148550; enrollment criteria listed in Supplemental Materials, http://links.lww.com/HC9/A721). Serum was also obtained from age-matched and sex-matched cases of Alagille syndrome (all molecularly confirmed) and α1-antitrypsin deficiency liver disease [LOGIC study (NCT00571272)], and biliary atresia from PROBE (young infants) and BASIC (older infants and children) studies (NCT00061828 and NCT00345553). Serum from liver biopsy–confirmed pediatric cases of NASH was obtained from a separate biorepository at Children's Hospital Colorado. Clinical information for participants included age, sex, race and ethnicity, BMI, serum aminotransferases, γ-glutamyl transferase, albumin, total bilirubin, prothrombin time expressed as international normalized ratio (INR), and platelet count. In addition, for the patients with MH, hemoglobin, white blood cell count, lactate, pyruvate, the genetic diagnosis (if obtained), acuity of presentation (acute or chronic), the involvement of other organ systems, and the time to either liver transplantation or death were included for analysis.

Assay methods and validation studies

Details about GDF15 and FGF21 assays are described in Supplemental Materials, http://links.lww.com/HC9/A721. Lactate, pyruvate, and alanine were measured as described (Supplemental Materials, http://links.lww.com/HC9/A721).[37]

Statistical analysis

The normality of distribution was first evaluated using Shapiro-Wilk and Kolmogorov-Smirnov statistics. The distribution of GDF15 and FGF21 deviated significantly from the normal distribution for all age groups (above and below age 6 mo). For consistency, nonparametric tests were generally used, even when log transformation approached normal distribution for certain subgroups. Descriptive values were expressed as the median, interquartile range (IQR), and entire range. A comparison of the biomarkers between different diagnostic groups was done by the Kruskal-Wallis test. Bonferroni correction was applied for pairwise comparisons. Differences between the distribution in the 2 classes was evaluated by the Mann-Whitney *U* test. The relationship between the biomarkers and various parameters of the patients was done by Spearman rank correlation. The diagnostic classification was evaluated using receiver operating characteristic (ROC) curves, and the AUC was reported, and comparisons were done using a paired sample nonparametric design. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated using a specific preassigned cutoff value, and the diagnostic OR was calculated.[38]

Kaplan-Meier plots were reported for survival to either liver transplantation or death and comparisons were evaluated by log-rank statistics. Cox proportional hazard models were used to assess whether GDF15 or FGF21 was associated with time-to-event after adjusting for important covariates in MH samples. Continuous variables were scaled to a mean of 0 and SD of 1 for ease of convergence. The model with all factors of interest (GDF15 or FGF21, age at sample, INR, acute vs. chronic MH, and genetically vs. nongenetically confirmed MH) was reported and the model with the lowest Akaike information criterion according to stepwise selection was reported. Interaction terms that had $p < 0.10$ were considered in the final multivariable model. The proportional hazards assumption was checked using Schoenfeld residual tests. GDF15 and FGF21 violated this assumption, so a $\log_{10}$ transformation was used. The variable inflation factor was assessed to ensure no multicollinearity issues in the final models.

Significance was set at 0.05. Statistical calculations were done using IBM SPSS Statistics version 28 (IBM), and R version 4.2.1.

RESULTS

Reference serum values for GDF15 and FGF21 in pediatric control subjects

Validation studies in healthy controls showed comparable results for serum and plasma for both GDF15 and FGF21 and a comparable distribution in controls was obtained on serum and plasma samples. Therefore, serum samples were used for this study. Validation studies are provided in Supplemental Materials, http://links.lww.com/HC9/A721.

In the hospital remnant serum samples used as control values, the evaluation of samples above the 90th percentile for GDF15 or FGF21 identified several recurrent causes of elevation, including renal dysfunction (most often nephrotic syndrome) and ongoing liver dysfunction, and in infants, prematurity, and hypoxic-ischemic injury. For use as control values, patient samples in these categories were excluded (Supplemental Figure S1, http://links.lww.com/HC9/A721). Of 186 included controls, 100 (54%) were male, and 28 (15%) were aged <6 months (Table 1). Upon graphical visualization, increased levels were noted for infants below 6 months for both GDF15 and FGF21 (Figure 1). Compared to children aged ≥6 months, infants aged <6 months had significantly higher GDF15 ($p < 0.001$) and FGF21 ($p = 0.019$). There was no

TABLE 1 Study participant populations

Variable	Controls	Mitochondrial hepatopathies	A1AT deficiency	ALGS	BA PROBE	BA BASIC	NASH
N	186	36	38	38	20	18	20
Male, N (%)	100 (54)	23 (64)	24 (63)	24 (63)	14 (70)	10 (56)	9 (45)
Age in months Median (IQR)	84 (14–158)	84 (9–112)	39 (12–99)	38 (12–102)	10.5 (3–34)	101 (49–182)	158 (128–174)
Age range in mo.	0.5–227	2–190	2–197	2–213	2–71	28–193	96–204
Age <6 mo, N (%)	28 (15)	6 (17)	2 (5)	7 (18)	7 (35)	0 (0)	0 (0)
Caucasian, N(%)	NA	26 (72.2)	35 (92.1)	26 (68.4)	12 (60)	9 (52.9)	7 (35)
Hispanic, N (%)	NA	7 (19)	3 (7.9)	11 (28.9)	6 (30)	4 (23.5)	17 (85)
BMI, M (IQR)	NA	16.3 (15.2–20.1)	16.3 (15.4–19.6)	16.4 (15.3–18.0)	16.3 (15.3–17.0)	16.8 (15.4–24.0)	31.2 (28.2–34.3)
AST, M (IQR), U/L	NA	84 (41–123)	83 (45–154)	157 (92–253)	129 (74–175)	62 (31–111)	112 (87–209)
T Bili, M (IQR), mg/dL	NA	0.5 (0.4–2.1)	0.5 (0.3–0.7)	2.9 (0.4–7.2)	1.9 (0.3–6.3)	0.5 (0.3–2.0)	NA
Albumin, M (IQR), g/dL	NA	3.8 (3.1–4.5)	4.3 (4.0–4.6)	4.1 (3.9–4.4)	3.9 (3.5–4.2)	4.2 (3.8–4.4)	NA
γ-GT, M (IQR), U/L	NA	94 (40–313)	59 (31–362)	371 (257–944)	275 (99–662)	107 (50–150)	54 (41–96)
INR, M (IQR)	NA	1.1 (1.0–1.9)	1.0 (0.9–1.1)	1.0 (1.0–1.1)	1.0 (0.9–1.1)	1.0 (0.99–1.0)	NA
Platelets, M (IQR), $\times 10^3$ per μL	NA	250 (144–361)	282 (243–353)	266 (193–244)	219 (183–395)	165 (103–240)	310 (278–348)
Lactate, M (IQR), mM	2.9 (1.5–4.7)[a]	2.1 (1.4–3.4)	1.9 (1.5–2.2)	2.0 (1.5–2.4)	1.6 (1.2–2.2)	2.0 (1.4–2.6)	NA
Pyruvate, M (IQR), μM	111 (80–221)	105 (81–122)	109 (92–139)	105 (82–134)	95 (76–106)	91 (77–114)	NA
Alanine, μM	373 (241–442)	288 (222–332)	227 (196–284)	281 (250–349)	247 (209–289)	269 (221–312)	NA
Lactate/pyruvate ratio	22.5 (17.5–30.0)	20.7 (15.0–27.4)	16.0 (13.7–21.1)	17.7 (14.3–23.4)	14.9 (14.0–23.0)	21.2 (13.8–28.6)	NA
Age ≥6 mo							
GDF15, M (IQR)	302 (248–399)	2666 (347–8788)	347 (254–525)	1136 (556–1663)	648 (343–1263)	460 (246–798)	319 (243–450)
Range	135–1756	253–107,266	191–4180	253–22,178	205–14,392	137–1266	172–891
# >98th percentile[a]	4/154 (2.6)	18/30 (62)	5/34 (15)	19/33 (58)	4/13 (31)	3/17 (18)	1/20 (5)
FGF21, M (IQR)	74 (35–115)	1006 (48–6368)	63 (33–120)	47 (27–85)	27 (5–62)	32 (23–42)	338 (128–338)
Range	11–925	19–37,724	7–1874	1–1556	<2–575	2–262	69–468
# >98th percentile[a]	4/155 (2.6)	18/30 (62)	3/34 (9)	3/33 (9)	1/13 (8)	0/17 (0)	4/20 (20)
Age <6 mo[b]							
GDF15, M (IQR)	708 (518–932)	29,201 (7165–48,044)		3704 (2489–4446)			NA
Range	398–2531	297–63,663		1575–5634			NA
# >98th percentile[a]	1/30 (3)	5/6 (83)		15/16 (94)			
FGF21, M (IQR)	91 (48–180)	11,057 (1,546–31,105)		144 (86–289)			NA
Range	38–1362	118–54,540		22–3299			NA
# >98th percentile[a]	1/28 (3.6)	5/6 (83)		1/16 (6.2)			NA

Note: Normal ranges: AST: 8–33 U/L, T Bili: 0.1–1.2 mg/dL, albumin: 3.4–5.4 g/dL, γ-GT: 5–40 U/L, INR <1.2, platelet count: 150–450$\times 10^3$/μL.

[a]N = 28. Number of samples above the 98th percentile of controls, which in children ≥6 months was for GDF15 >874 ng/L and for FGF21 >347 ng/L, and for infants <6 months was for GDF15 >2046 ng/L and for FGF21 >1123 ng/L.

[b]For age <6 months, due to the low number of participants, the groups A1AT, ALGS, and BA were merged to analyze the results of the biomarker proteins.

Abbreviations: A1AT, α1-antitrypsin deficiency; ALGS, Alagille syndrome; AST, aspartate aminotransferase; BA, biliary atresia; BMI, body mass index; γ-GT, γ-glutamyl transferase; GDF15, growth differentiation factor 15; INR, international normalized ratio; IQR, interquartile range; M, median; NA, not available; T Bili, total bilirubin.

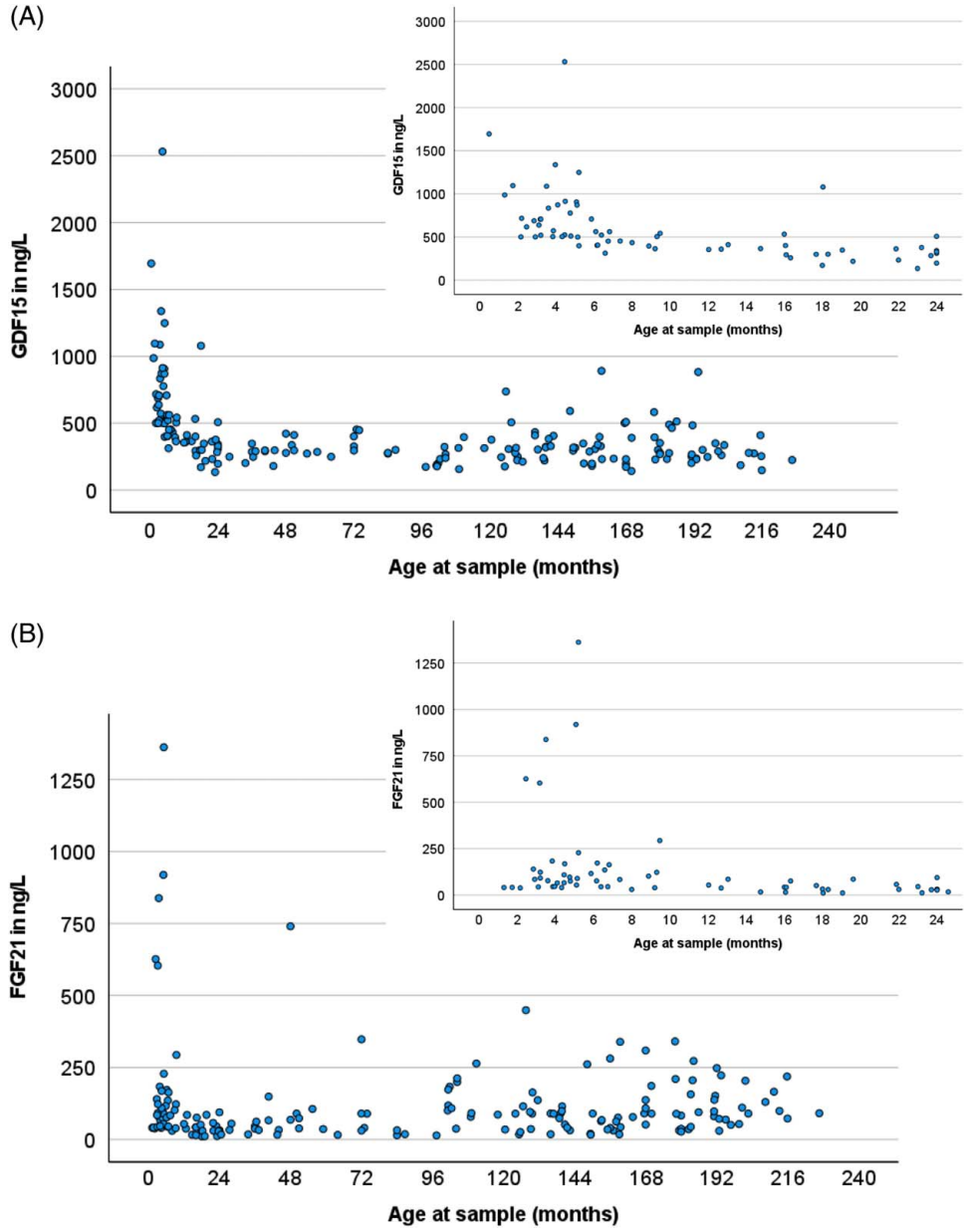

FIGURE 1 Biomarkers by age in normal controls. Values of biomarkers (A) GDF15 and (B) FGF21 in normal controls as a function of age (months). Inserts illustrate an increase in values in infants aged <6 months. Occasional outliers are also noted. Abbreviation: GDF15, growth differentiation factor 15.

difference by sex for either biomarker. For age ≥6 months, GDF15 and FGF21 had a clinically insignificant correlation with age (Spearman $\rho = -0.186$, $p = 0.02$ for GDF15 and Spearman $\rho = 0.326$, $p < 0.001$ for FGF21). Values in infants aged <6 months did not show a significant relation with age. Samples obtained from fasting children waiting for gastrointestinal endoscopy had slightly lower values than hospital remnant samples, but this difference was not statistically significant ($p = 0.135$ for GDF15 and $p = 0.08$ for FGF21). Given multiple substantive outliers, particularly in young infants, for diagnostic evaluation, the 98th percentile was used: for infants <6 months this was 2046 ng/L for GDF15 and 1123 ng/L for FGF21; for children age ≥6 months this was 874 ng/L for GDF15 and 347 ng/L for FGF21.

Baseline characteristics of MH and liver disease cohorts

Participant groups in this study are described in Table 1. Serum samples were obtained from 36 children with a clinically diagnosed MH as per the MITOHEP protocol criteria (described in Supplemental Materials, http://links.lww.com/HC9/A721), which included 17

participants with molecularly proven diagnosis (Supplemental Table S1, http://links.lww.com/HC9/A721), of which 14 had a defect in mtDNA maintenance or a mtDNA deletion.[7] Of the 36 participants, 6 presented acutely and 30 had chronic liver disease, 14 also had neurological symptoms and 12 had muscle symptoms. Lactate was elevated above postprandial levels in 57% of participants (Supplemental Table S2, http://links.lww.com/HC9/A721).

Serum was obtained from 38 participants with each of the following diagnoses: α1-antitrypsin deficiency, Alagille syndrome, and biliary atresia matched to MH for age, sex, and BMI where possible (Table 1). Patients with biliary atresia were derived from 2 studies: 18 participants from the BASIC study (enrolled at age ≥6 mo) and 20 patients in the PROBE study (enrolled before age 6 mo). The PROBE participants were significantly younger in age and had significantly more liver dysfunction as reflected by total bilirubin and other liver biochemistries. The 20 participants with NASH had a higher percentage of Hispanic ethnicity compared to the other groups, and as expected for this condition, the BMI was significantly elevated (Table 1).

GDF15 and FGF21 concentrations are increased in MH

In participants aged ≥6 months, there were statistically significant differences in GDF15 levels across the various groups ($p < 0.001$) (Figure 2 and Table 1). Levels of GDF15 were markedly increased in patients with MH (MH median increased 8.8-fold × median of controls, $p < 0.001$) (Figure 2A), and moderately raised in those with Alagille syndrome (median increased 3.8-fold × median of controls, $p < 0.001$) and young patients with biliary atresia (median increased 2.1-fold × median of controls, $p < 0.001$) (Figure 2B). For those ≥6 months old, there were also statistically significant differences in FGF21 levels across the diagnostic groups ($p < 0.001$) (Figure 2C and Table 1). Levels of FGF21 were markedly raised in those with MH (median

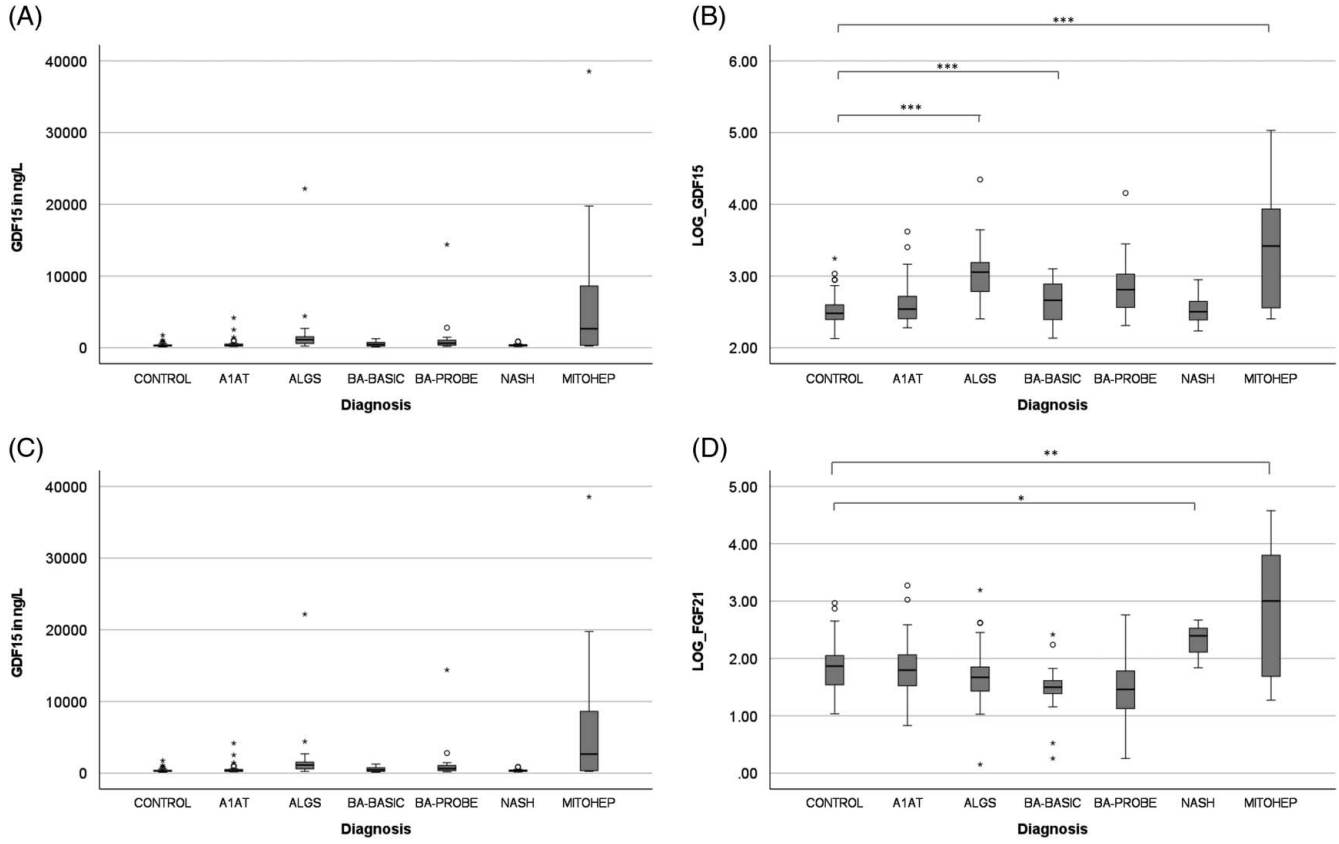

FIGURE 2 Biomarkers in different categories of study participants ages 6 months to 18 years. Levels of biomarkers for patients ages 6 months to 18 years. (A) The levels of GDF15 (ng/L) are significantly increased in patients with MH compared to all other diagnostic groups and controls. (B) The $\log_{10}$-transformed GDF15 levels better visualize the moderate increase of GDF15 levels in patients with Alagille syndrome and biliary atresia from PROBE. (C) The levels of FGF21 (ng/L) are significantly increased in patients with MH compared to all other diagnostic groups and controls. (D) The $\log_{10}$-transformed FGF21 levels better visualize the moderate increase of FGF21 levels in patients with NASH. Abbreviations: A1AT, α1-antitrypsin deficiency; ALGS, Alagille syndrome; BA-BASIC = biliary atresia from the BASIC study; BA-PROBE, biliary atresia from the PROBE study; GDF15, growth differentiation factor 15; MITOHEP, mitochondrial hepatopathies. *$p < 0.05$, **$p < 0.01$, ***$p \leq 0.001$.

increased 13.6-fold × median of controls $p < 0.01$) (Figure 2C), and moderately raised in patients with NASH (median increased 4.6-fold × median of controls, $p = 0.02$) (Figure 2D). The values of both biomarkers were significantly higher than both the normal controls and the other liver disease groups in both age groups (Supplemental Table S3, http://links.lww.com/HC9/A721).

In infants aged <6 months, because of limited numbers, the Alagille syndrome, α1-antitrypsin deficiency, and biliary atresia groups were combined into one group called Other Liver Diseases. There were statistically significant differences for GDF15 levels by disease groups (Kruskal-Wallis $p < 0.001$) (Figures 3A, B) with markedly increased levels in MH (median increased 41.2-fold × the median of controls) and a milder increase in other liver diseases (median increased 5.2-fold × median of controls) (pairwise comparisons Bonferroni-adjusted statistically significant for these 2 groups at $p < 0.001$). There were also statistically significant differences for FGF21 levels by disease groups ($p = 0.002$) (Figures 3C, D) with markedly increased values in MH (median increased 121-fold × the median of controls, $p = 0.001$) and increased values to a lesser degree in other liver diseases (median increased 1.6-fold × median of controls, $p = 0.039$).

Combining age groups, for GDF15, 2.7% of controls exceeded the 98th percentile of the control range, while in other liver diseases combined 35.3% exceeded the 98th percentile, and in MH 63.9% of cases exceeded this value. For FGF21, 2.7% of controls exceeded the 98th percentile of the control range, in MH 63.9% exceeded this value, and in other liver diseases, 9.0% exceeded it (Chi-square $p < 0.001$ for each marker).

Relationship of the biomarkers with clinical variables in MH

If the driver of these biomarkers is the mitochondrial stress response in patients with MH, then a similar increase in both biomarkers in the same patients would be expected. Indeed, in patients with MH, there was a strong correlation between GDF15 and FGF21 serum

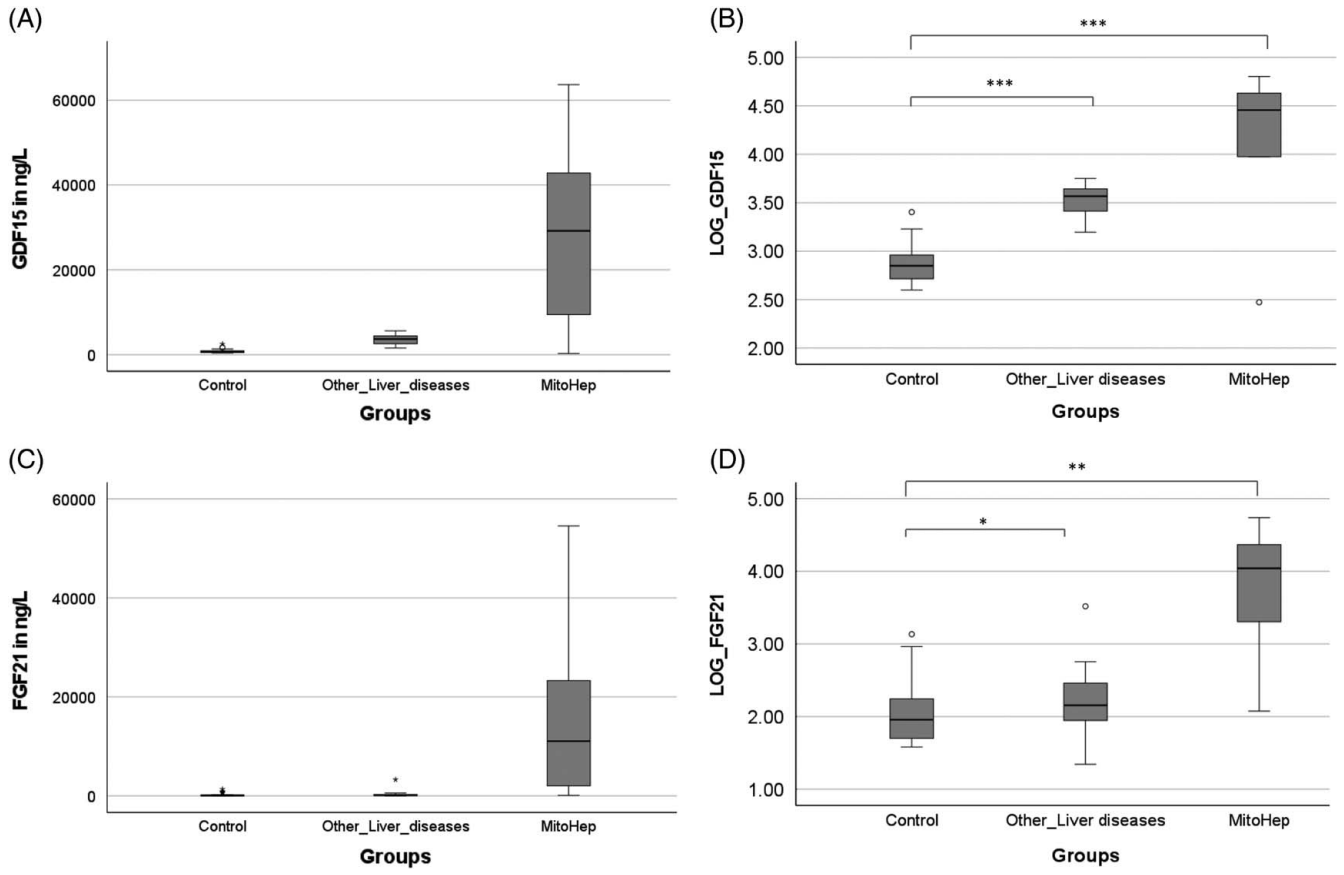

FIGURE 3 Biomarkers in different categories of study participants ages <6 months. Levels of biomarkers for patients aged <6 months. (A). Levels of GDF15 are significantly increased in patients with MH and (B) log10 of GDF15 shows a moderate increase in other liver diseases. (C) Levels of FGF21 are significantly increased in patients with MH and (D) log10 of FGF21 shows that no significant increase was present in other liver diseases. Abbreviations: GDF15, growth differentiation factor 15; MITOHEP, mitochondrial hepatopathies. *$p < 0.05$, **$p < 0.01$, ***$p \leq 0.001$.

concentrations ($\rho = 0.87$, $p < 0.01$). This correlation was not present in controls or other liver disorders ($\rho = 0.17$). Patients with genetically confirmed MDs (N = 17) had higher levels of both biomarkers than those without a confirmed molecular MH etiology (N = 19): for GDF15 median = 9283 ng/L (IQR: 3752–19,435) versus 554 (IQR: 297–3878), $p = 0.003$, and for FGF21 median = 6046 ng/L (IQR: 2361–12,596) versus 192 (IQR: 42–1697), $p = 0.008$. In contrast, there was no difference in biomarkers between patients with MH who presented acutely (N = 6) versus those who presented with a chronic condition (N = 30) (GDF15: $p = 0.268$, FGF21: $p = 0.394$). There was a moderate negative correlation with age for GDF15 (Spearman $\rho = -0.635$, $p < 0.001$), and for FGF21 ($\rho = -0.612$, $p < 0.001$). There were no significant differences in either biomarker by sex, ethnicity, or race.

Surprisingly, there was no significant correlation in MH participants of either biomarker with clinically measured serum lactate, pyruvate, or the lactate/pyruvate molar ratio (for both GDF15 and FGF21 all $p \geq 0.2$), but FGF21 had a weak positive correlation with pyruvate measured in this study ($\rho = 0.5$, $p = 0.002$) and with lactate ($\rho = 0.38$, $p = 0.019$). Within MH, there were weak to moderate correlations of the biomarkers with various indicators of the severity of liver dysfunction. There was a moderate positive correlation between aspartate aminotransferase and GDF15 ($\rho = 0.678$, $p < 0.001$), and FGF21 ($\rho = 0.659$, $p < 0.001$), and with signs of liver dysfunction, including a negative correlation with albumin (GDF15: $\rho = -0.584$, $p < 0.001$ and for FGF21: $\rho = -0.626$, $p < 0.001$) and a positive correlation with INR (GDF15: $\rho = 0.505$, $p = 0.007$; FGF21: $\rho = 0.398$, $p = 0.040$). There was also a moderate negative correlation with hemoglobin levels (GDF15: $\rho = -0.687$, $p < 0.001$, and FGF21: $\rho = -0.631$, $p < 0.001$), but not with the platelet count. Within MH, the correlation with total bilirubin was weak (GDF15: $\rho = 0.484$, $p = 0.003$; FGF21: $\rho = 0.426$, $p = 0.011$) and not significant with γ-glutamyl transferase levels. In contrast in other liver disorders (biliary atresia, α1-antitrypsin deficiency, and Alagille syndrome), GDF15 did relate moderately to cholestasis-related markers: for total bilirubin ($\rho = 0.739$, $p < 0.001$) and γ-glutamyl transferase levels ($\rho = 0.708$, $p < 0.001$), but not FGF21, indicating that a different process was likely driving GDF15 secretion in these other liver diseases.

The diagnostic utility of the biomarkers to identify MHs

Both biomarkers showed equally good discrimination on ROC analysis between clinically diagnosed MH versus controls and all other liver disorders (Figure 4A): GDF15: AUC = 0.779 ± 0.049, 95% CI: 0.682–0.876, and FGF21: AUC = 0.778 ± 0.056, 95% CI: 0.669–0.887), difference not significant $p = 0.98$. We next examined the performance of the biomarkers to identify molecularly diagnosed MH (Figure 4B). In patients with genetically confirmed MH, GDF15 and FGF21 showed even better discrimination from other liver diseases: GDF15: AUC = 0.930 ± 0.042 (95% CI: 0.846–1.000), and FGF21: AUC = 0.904 ± 0.059 (95% CI: 0.789–1.000), difference between AUC of markers not significant $p = 0.34$. A combination of GDF15 and FGF21 either as a scaled sum or product did not improve the AUC. Both markers performed much better than lactate, pyruvate, alanine, or the lactate/pyruvate ratio (Supplemental Figure S2, http://links.lww.com/HC9/A721).

Next, we examined the clinical performance for diagnosis using a cutoff at the 98th percentile of controls; for infants aged <6 months GDF15 cutoff was 2046 ng/L and for children ≥6 months 874 ng/L, and for FGF21 for infants <6 months 1123 ng/L and for children ≥6 months 374 ng/L. We combined the results from these 2 age groups (Table 2), showing that GDF15

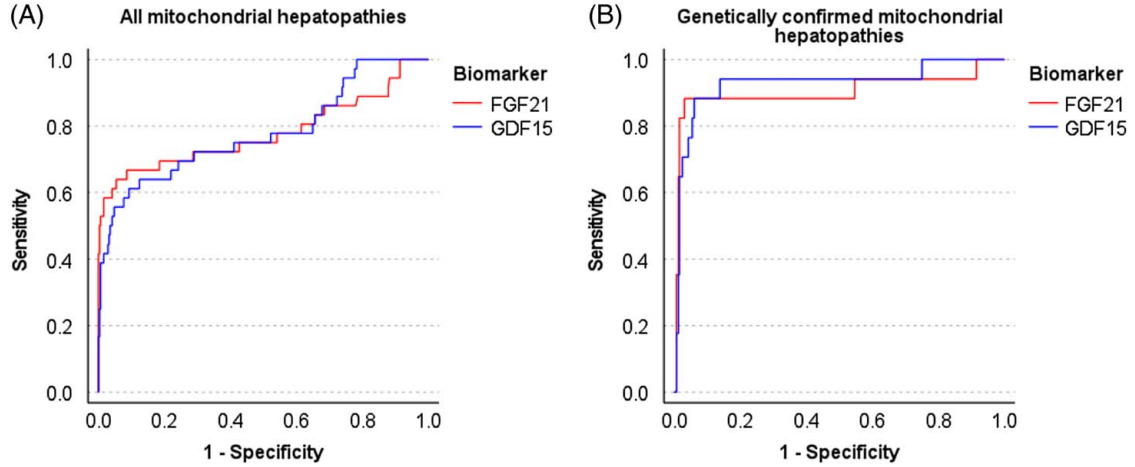

FIGURE 4 Receiver operating curve analysis of the biomarkers. The receiver operating curves of both GDF15 and FGF21 are shown for (A) mitochondrial hepatopathies compared to all other liver diseases and patients, and (B) the genetically confirmed mitochondrial hepatopathies compared to all other subjects. Abbreviation: GDF15, growth differentiation factor 15.

TABLE 2 Diagnostic performance of biomarkers

Marker	Positive cases	Comparative cases	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	DOR
All clinically diagnosed mitochondrial hepatopathies							
GDF15	36	317	64	84	31	95	9.0
FGF21	36	315	64	95	58	96	31.0
GDF15 and FGF21	36	315	61	98	76	96	69.1
Molecularly diagnosed mitochondrial hepatopathies							
GDF15	17	336	94	82	21	100	75.1
FGF21	17	334	88	93	38	99	92.7
GDF15 and FGF21	17	334	88	96	52	99	171.4

Note: The diagnostic performance for the biomarkers is given for values exceeding the 98th percentile of controls. GDF15 and FGF21 indicate that both markers exceed the threshold. The values of sensitivity, specificity, positive, and negative predictive value are calculated for the study population of this study without further adjustment for the prevalence in the overall clinical population, which is not well defined.
Abbreviations: DOR, diagnostic odds ratio; GDF15, growth differentiation factor 15; NPV, negative predictive value; PPV, positive predictive value.

and FGF21 had similar sensitivity, but FGF21 had higher specificity to identify clinically diagnosed MH resulting in a higher diagnostic OR. Given the higher values in patients with molecularly diagnosed MH, the sensitivity of both biomarkers increased in that subgroup without much loss in specificity. Since most patients with MH had both biomarkers elevated, but because GDF15 increased in some cholestatic conditions whereas FGF21 only increased somewhat in NASH, we evaluated the performance of the elevation of both biomarkers together. Having both biomarkers above the 98th percentile resulted in only a limited decrease in sensitivity (from 64% to 61%) but a marked increase in specificity and positive predictive value resulting in an improved diagnostic OR. This was particularly true for molecularly diagnosed MH with a sensitivity of 88%, specificity of 96%, a good positive predictive value (52%), and an excellent negative predictive value (99%) in the setting of this study population, making it the clinical test with the greatest diagnostic OR of 171.4, a comparative indicator of test performance.[38]

Elevations of GDF15 and FGF21 in MH were often well above those of other liver diseases,[24] and thus a very high level may confer a particularly strong indication for MH. For instance, using a high cutoff value of 6000 ng/L for GDF15 identified no controls from this study including no sporadic high levels except for only 1 patient with severe renal disease; however, it identified 39% of clinically diagnosed MH and 65% of molecularly proven MH, whereas it only identified 1 case each with Alagille syndrome and biliary atresia, making this threshold a very specific indicator of MH.

Predictors of outcome in MHs

Of 36 patients with clinically diagnosed MH, 3 patients underwent liver transplantation and 12 died, representing a 42% adverse outcome rate, and for this analysis the term event-free survival (EFS) applies to time-to-transplant or death, whichever was first. Median follow-up time was 11 months (IQR: 0.6–37.2). EFS with native liver (survival without liver transplant) was significantly worse in patients with acute MH presentation [estimated 2-year EFS: 20% (95% CI: 3%–100%)], compared to chronic presentation [65% (95% CI: 49%–86%), $p < 0.001$; Figure 5A]. EFS was worse in patients with an identified genetic cause [2-year EFS: 42% (95% CI: 23%–76%)] compared to those without a recognized genetic cause [74% (95% CI: 55%–100%), $p = 0.026$] (Figure 5B). There was no EFS difference by sex ($p = 0.7$), ethnicity ($p = 0.19$), or by the presence of neurological, muscular, or cardiac symptoms.

In univariate Cox proportional hazard modeling, factors associated with EFS included aspartate aminotransferase ($p = 0.002$), albumin ($p < 0.001$), and INR ($p < 0.001$) for patients with clinically diagnosed MH. Lactate was not significantly associated with EFS ($p = 0.8$), but with age ($p = 0.008$). These same factors were also significant within the group of genetically diagnosed MH where the INR had the strongest significance ($p < 0.001$).

A multivariable Cox PH model was examined that included scaled INR, scaled GDF15, scaled age at the sample, the acuteness of presentation, and the presence of a recognized genetic cause. After adjusting for age, acute versus chronic presentation, and genetic cause identified or not, only 2 predictors remained significantly associated with EFS: the INR ($p = 0.04$) and the level of GDF15 ($p = 0.007$) (Table 3). For every SD increase in $\log_{10}$ GDF15, that is, an 8-fold increase, the hazard of death or transplant increased 9.62-fold (95% CI: 1.8–50.4), and for every SD increase in the INR, that is, a 0.73 increase, it increased 3.5-fold (95% CI: 1.1–11.7). Using stepwise selection, the best set of covariates that resulted in the lowest Akaike information criterion were GDF15 and INR. In this model, both predictors remained significantly associated with time-to-event ($p = 0.005$ and $p = 0.0009$, respectively).

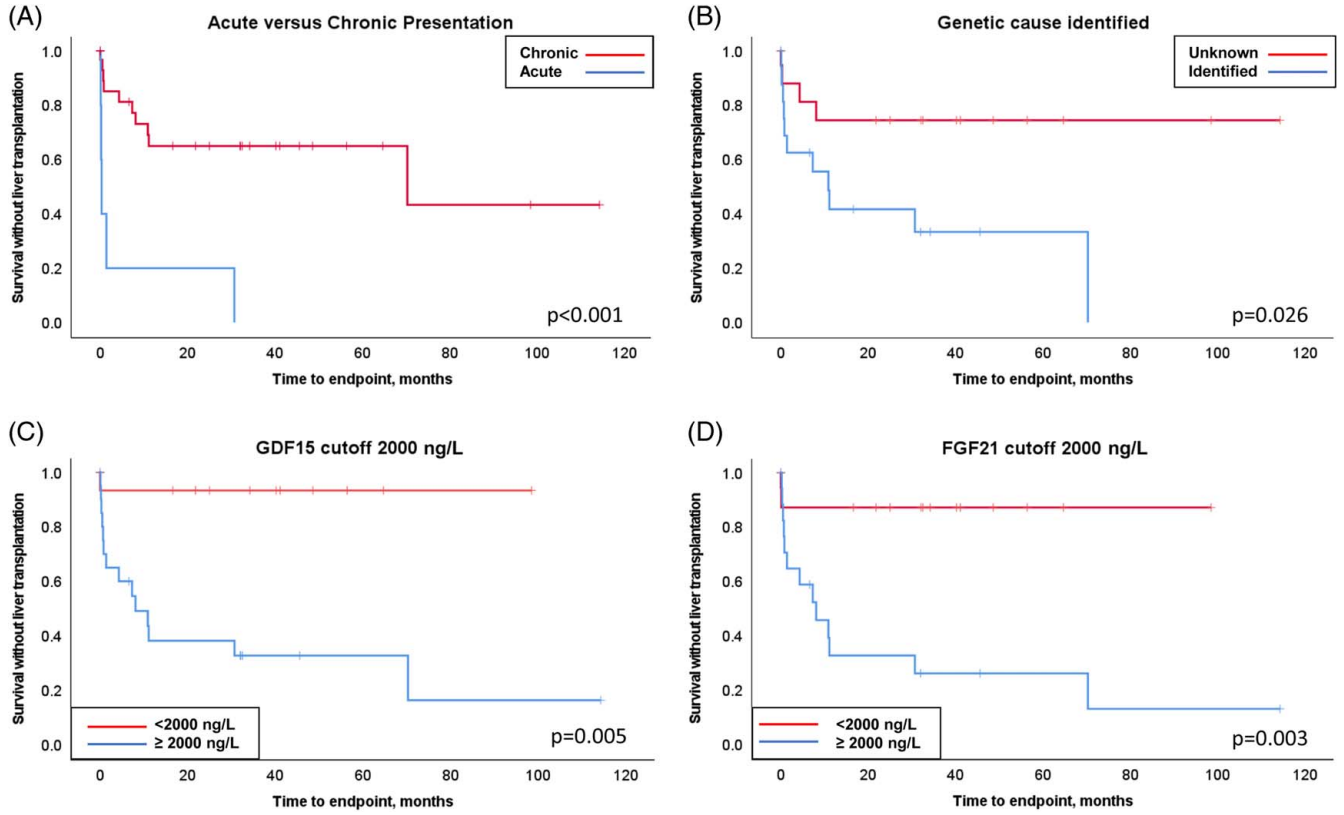

FIGURE 5 Kaplan-Meier plot of survival without liver transplantation in patients with mitochondrial hepatopathy. Kaplan-Meier plots are shown for survival without liver transplantation (EFS) for participants with mitochondrial hepatopathy. (A) EFS is worse in patients with acute (blue, N = 6, 5 events) vs. chronic presentation (red, N = 30, 10 events). (B) EFS is worse for patients with a known genetic diagnosis (blue, N = 17, 11 events) vs. those without a known genetic diagnosis (red, N = 19, 4 events). (C) Significantly decreased EFS was demonstrated for patients with a GDF15 > 2000 ng/L (blue, N = 21, 14 events) vs. patients with GDF15 <2000 ng/L (red, N = 15, 1 event). (D) Significantly decreased EFS was demonstrated for patients with FGF21 > 2000 ng/L (blue, N = 18, 13 events) vs. those with <2000 ng/L (red, N = 16, 2 events). Abbreviations: EFS, event-free survival; GDF15, growth differentiation factor 15.

A similar multivariable model was developed with FGF21, and similarly, the biomarker FGF21 and INR remained significant predictors. Using stepwise selection, the best set of covariates that resulted in the lowest Akaike information criterion were FGF21 and INR. In this model, both predictors remained significantly associated with time-to-event at $p = 0.02$ and $p = 0.0002$, respectively. The strong correlation of GDF15 and FGF21 in this patient population does not allow a model with both biomarkers. The 3 significant variables did not show differences in discriminating between those who survived event-free with native liver versus those not according to AUC on ROC analysis: INR (0.889 ± 0.067), GDF15 (0.835 ± 0.060), and FGF21 (0.835 ± 0.077) were not significantly different.

For a clinical application, defining a cutoff value to predict outcomes would be useful. The 2-year EFS rate (survival without liver transplant) for those with GDF15 < 2000 ng/L was 93% (95% CI: 80%–100%), compared to 38% (95% CI: 22%–68%) for patients with a GDF15 level above this threshold, $p = 0.005$ (Figure 5C). This difference was also present in the molecularly diagnosed MH. With a cutoff of 2000 ng/L for FGF21, a similar strong difference in outcome was noted with a 2-year EFS of 87% (95% CI: 71%–100%), in patients with less than 2000 ng/L compared to 33% (95% CI: 16%–66%) in those over 2000 ng/L, $p = 0.003$ (Figure 5D).

DISCUSSION

Our study showed that both GDF15 and FGF21 concentrations are markedly elevated in MH, much more so than for several other pediatric liver disorders. Both biomarkers tend to elevate in the same patients, and in this diagnostic group are markedly intercorrelated, likely reflecting that their elevation is the consequence of the activation of the universal mitochondrial stress response. An intercorrelation of both markers has been noted before, albeit not to this degree.[13,24,26,28,29] The elevation was even more pronounced in molecularly proven MH, the vast majority of which have mtDNA depletion disorders or mtDNA deletions, both of which have previously been implicated as inducing the strongest stress response.[20,24,25] Elevations of these biomarkers in liver

TABLE 3 Cox proportional hazard model for survival without liver transplantation in patients with mitochondrial hepatopathies

Full model	Standard deviation (continuous) or reference (categorical)	GDF15 model Hazard ratio (95% CI); *p*	FGF21 model Hazard ratio (95% CI); *p*
Scaled Log_{10} (biomarker)	GDF15 = 0.81, FGF21 = 1.1	9.62 (1.84–50.38); $p = 0.0073$	11.61 (1.2–112.01); $p = 0.034$
Scaled age at sample (months)	63 mo	0.99 (0.15–6.59); $p = 0.9884$	4.28 (1.57–11.69); $p = 0.0045$
Scaled INR	0.73	3.51 (1.06–11.65); $p = 0.0398$	2.6 (0.47–14.33); $p = 0.272$
Acute	Chronic	2.99 (0.57–15.79); $p = 0.1965$	1.14 (0.19–7.06); $p = 0.885$
Genetic cause known	Unknown	1.31 (0.18–9.37); $p = 0.7897$	11.61 (1.2–112.01); $p = 0.034$
Best fitting model			
Scaled Log_{10} (biomarker)	GDF15 = 0.81, FGF21 = 1.1	7.16 (1.82–28.06); $p = 0.0048$	7.95 (1.38–45.85); $p = 0.0204$
Scaled INR	0.73	4.7 (1.89–11.68); $p = 0.0009$	5.28 (2.18–12.82); $p = 0.0002$

Note: The results from multivariable Cox proportional hazard models. The first column are the models that included GDF15 as the primary predictor, and the second column included FGF21 as the primary predictor. Continuous variables were scaled (mean = 0, SD = 1) for ease of convergence.
Abbreviations: GDF15, growth differentiation factor 15; INR, international normalized ratio.

MDs were occasionally reported.[20,24] In our study, cholestatic conditions such as biliary atresia and Alagille syndrome had moderate elevations of GDF15, but to a much lesser degree than in MH, and correlated with markers of cholestasis (total bilirubin and γ-glutamyl transferase levels). Elevation in other cholestatic conditions in adults, such as primary biliary cholangitis and primary sclerosing cholangitis, had been noted.[24,33] In contrast, in these cholestatic conditions, FGF21 was not elevated. This likely implies that the transcriptional process driving the increase in GDF15 in cholestasis was likely different from the mitochondrial stress response, although some likely secondary mitochondrial abnormalities and cell death pathways may be induced by accumulated bile acids in cholestatic liver diseases[39–41] and have been reported in biliary atresia.[42] Similarly, FGF21, but not GDF15, was moderately increased in NASH, as reported.[24,35,36] As a result, the combined elevation of both GDF15 and FGF21 present in MH, because it is driven by a mitochondria-specific process, provided specificity in the context of liver disease.

For use in a clinical setting, an appropriate clinical cutoff must be identified. Given the number of outliers in the control population, the 98th percentile was chosen. Insufficient specificity was obtained when using the 95th percentile, which had been used in several previous publications.[21,22,28,29,33] Moreover, a careful review of control data identified that values are higher in infants younger than 6 months of age. Fortunately, young infants affected by MH also had much higher values, still allowing diagnostic differentiation. Correct identification of the 98th percentile requires the analysis of a sufficient number of controls, since for every 100 controls the 98th percentile is only identified from 2 data points. Using the 98th percentile provided a reasonable identification of MH with an AUC of 0.78 for both biomarkers and above 0.91 for molecularly proven MH cases. However, because the elevation for both markers together is specific to MH, and not present in the other liver disease categories or even in other noncontrol samples such as with renal disease, the elevation of both biomarkers substantially increased the diagnostic value as reflected in a strong diagnostic OR. The diagnostic recognition was particularly strong for those patients with a molecular MH diagnosis. If both biomarkers were elevated, then the positive predictive value of over 50% would certainly warrant extensive molecular investigations and, if clinically indicated and feasible, may further justify proceeding with enzymatic diagnosis on a liver biopsy. Expanding the dynamic range of the markers to very high levels provided further useful information as it could identify patients with a very strong indication for MH.

The elevation of these biomarkers in other clinical situations has raised doubt about the value of these protein biomarkers for the identification of primary MDs when used in a wide multisystemic indication.[34] Within the clinical context of mitochondrial myopathies of both skeletal muscle and ocular muscle, multiple studies have shown good evidence for the diagnostic value of both biomarkers GDF15 and FGF21.[13,20–22,24,29–31] Our study indicates that these biomarkers also have significant value in the context of the differential diagnosis of pediatric liver diseases to recognize MH, with an ROC AUC of more than 0.9 for genetically confirmed MH, similar to that of myopathies. It further illustrates in this clinical context the increased specificity when using a combination of both markers. This biomarker has effective diagnostic value for

MDs in the context of pediatric liver diseases, where elevated lactate has been shown to be an integral feature of any cause of acute liver failure, and the lactate:pyruvate ratio did not provide discrimination for MH. In the context of liver disease, elevated lactate not only can reflect redox imbalance of NADH, but also interruption to the Cori cycle due to hepatocyte dysfunction and is thus not specific to mitochondrial dysfunction by primary mitochondrial disease. In the current study, serum lactate, pyruvate, and L:P ratio similarly were poor biomarkers for mitochondrial disease in this pediatric cohort with acute and chronic liver disease. Our study examined children with the most common cholestatic liver disorders and NASH as disease controls, thus expanding this study to other liver diseases may be helpful, specifically including infants and children presenting with other causes of acute liver failure.[43] A study reviewing these biomarkers specifically in the context of pediatric patients with acute liver failure is indicated. Only about half of clinically diagnosed MH collected over the course of over 15 years in the MITOHEP study had a molecular diagnosis, which is nowadays considered the gold standard for the diagnosis of a primary MDs.[44] Our findings confirmed and were stronger in this specific subgroup, thus supporting the broad use of genotyping in suspected MH. Given the difficulty of estimating the relative incidence of MHs encountered in various clinical settings (acute or chronic pediatric liver disease), the predictive values reported here are for the patients enrolled in this study, without such population adjustment and represent a limitation of this study.

Importantly, in addition to their diagnostic value, the biomarkers also provided prognostic information. The severity of liver dysfunction, as reflected by the INR, and GDF15 and FGF21 each independently were predictive of the likelihood of liver transplantation or death. Given the marked intercorrelation of the 2 biomarkers, both provided similar prognostic information, although the statistical strength was slightly larger for GDF15. The ability of the biomarkers to predict survival without liver transplant was similar (ROC AUC) to that reported for the Liver Injury Unit scoring system.[45] From a clinical perspective, a cutoff of 2000 ng/L for each biomarker provided strong predictive power whereby for GDF15 the chances of death or transplant increased from 6.7% to 66.7% (a 10-fold increase) and for FGF21 from 12.5% to 81.2% (a 6.5-fold increase). This distinction was equally present in molecularly proven MH, although the small number of patients did not allow statistical analysis. This is the first study that provided prognostic value for these biomarkers in the context of primary MD. FGF21 has previously been related to long-term complications in organic acidurias.[37] Very few markers are available to monitor or predict disease progression in MD.[46] Other studies showed the relationship between the biomarkers with MD severity,[21,23,26,28,29] but not with disease progression.[23,32] The strong prognostic value of the identified markers will have implications for clinical decision-making, particularly if they can be replicated in an independent study of MH. Given the performance of GDF15 and FGF21 in predicting liver-related endpoints in MH and their relationship to mitochondrial stress response, both markers might be explored in the future as surrogate endpoints for clinical trials of novel therapeutics for MH.

In conclusion, this study indicates that the combination of elevation of both GDF15 and FGF21 is useful for identification of MH, particularly molecularly proven MH, in the context of pediatric liver disease, using age-adjusted cutoff values with a diagnostic OR that exceeds those of previous studies in other clinical contexts.[23,33,47] Expanding these diagnostic findings in a study of additional liver diseases, including acute liver failure, will help to validate the utility of these markers in clinical practice. Further, additional biomarkers such as gelsolin, inhibin E, HGF, SCGF-β, sE-selectin, or metabolomics markers should be investigated to determine if their inclusion will add to the diagnostic utility.[19,48,49]

AUTHOR CONTRIBUTIONS

Study concept and design: Johan L.K. Van Hove, Marisa W. Friederich, and Ronald J. Sokol. Laboratory studies: Marisa W. Friederich, Dana K. Strode, Roxanne A. Van Hove, Rohit Sharma, and Hardik Shah. Contribution of samples and clinical data: Marisa W. Friederich, Linda Gabel, Simon Horslen, Rohit Kohli, Mark A. Lovell, Alexander G. Miethke, Jean P. Molleston, Philip Rosenthal, Rene Romero, James E. Squires, Rohit Sharma, Estella M. Alonso, Stephen L. Guthery, Binita M. Kamath, Kathleen M. Loomes, Philip Rosenthal, Krupa R. Mysore, Laurel A. Cavallo, Pamela L. Valentino, Shikha S. Sundaram, John C. Magee, and Ronald J. Sokol. Data and statistical analysis: Johan L.K. Van Hove and Krupa R. Mysore. First draft writing: Johan L.K. Van Hove, Marisa W. Friederich, Jane Estrella, and Ronald J. Sokol. Critical rewriting: all authors. Leadership and funding: Johan L.K. Van Hove and Ronald J. Sokol. Final responsibility and communicating authors: Johan L.K. Van Hove and Ronald J. Sokol.

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CONFLICTS OF INTEREST

Ronald J. Sokol is on advisory committees of Mirum and Albireo and consults with Astellas. Johan L.K. Van Hove has been an advisor to Stealth Biotherapeutics. Philip Rosenthal receives research support from Abbvie, Albireo, Arrowhead, Gilead, Merck, Mirum, Takeda, and Travere, and is a consultant for Albireo, Ambys, Audentes, BioMarin, Dicerna, Encoded, MedinCell, Mirum, Takeda, and Travere. Kathleen M. Loomes participated in advisory committees for Mirum and Albireo, and is a consultant for Travere Therapeutics. Binita M. Kamath is a consultant of Mirum, Albireo, and Audentes, and has unrestricted education grants from Mirum and Albireo. Rene Romero is a consultant for Mirum and Albireo and has a clinical trial with Gilead. Jean P. Molleston receives research funding from Albireo, Abbvie, Mirum, Gilead, and the CF Foundation. Rohit Sharma has equity in bluebird bio. Simon Horslen consults for Albireo, Ipsen and iEcure and received grants from Mirum. Rohit Kohli consults and advises Mirum, Albireo, Sanofi, Epigen, and Intercept. Alexander G. Miethke consults and received grants from Mirum. Pamela L. Valentino is on the speakers' bureau for Mirum. Shikha S. Sundaram advises Mirum and Albireo. The remaining authors have no conflicts to report.

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