https://doi.org/10.31925/farmacia.2025.1.10 **ORIGINAL ARTICLE**

THE ROLE OF SOME PROTEIN BIOMARKERS IN THE EARLY IDENTIFICATION OF THE RISK OF CYSTIC FIBROSIS ASSOCIATED LIVER DISEASE

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Abstract

The study aimed to evaluate the presence of three protein biomarkers in the serum of patients with cystic fibrosis (CF) and correlate these markers with the degree of hepatic impairment. We conducted a cross-sectional observational study that included 36 paediatric patients with CF, equally divided into two groups (a Case group - with hepatic involvement and a Control group), recruited between November 2023 and March 2024, in accordance with the new guidelines of the CF Foundation. The following biomarkers were measured in the serum of these patients: ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), Endoglin/CD105 and Tissue Inhibitor of Metalloproteinases-4 (TIMP-4). Serum TIMP-4 levels were significantly elevated in patients with hepatic involvement, and this finding correlated with the calculated cystic fibrosis scores. No significant differences were observed in the levels of Endoglin and UCHL1 between the two groups. TIMP-4 is a non-invasive, rapid and cost-effective biomarker associated with the early onset of hepatic lesions.

Rezumat

Studiul a evaluat prezența a trei biomarkeri proteici în serul pacienților cu fibroză chistică (FC) și corelează acești markeri cu gradul de afectare hepatică. Acest studiu a fost observațional transversal și a inclus 36 de copii cu FC, împărțiți egal în două grupuri (grupul Case - cu afectare hepatică și grupul de Control), recrutați în perioada noiembrie 2023 – martie 2024, conform noului ghid al Fundației pentru FC. În serul acestora au fost măsurați următorii biomarkeri: hidrolaza terminală a ubiquitinei carboxil (UCHL1), Endoglin/CD105 și Inhibitorul Tisular al Metaloproteinazelor-4 (TIMP-4). Nivelurile serice de TIMP-4 au fost semnificativ crescute la pacienții cu afectare hepatică, acest rezultat corelându-se cu scorurile de fibroză chistică. Endoglinul și UCHL1 nu au prezentat diferențe semnificative între cele două grupuri. TIMP-4 este un biomarker proteic neinvaziv, rapid și accesibil din punct de vedere al costurilor, care se asociază cu apariția timpurie a leziunilor hepatice.

Keywords: ubiquitin carboxyl-terminal hydrolase L1, Endoglin, Tissue Inhibitor of Metalloproteinases-4, cystic fibrosis-related liver disease

Introduction

Cystic fibrosis (CF) or mucoviscidosis is a monogenic autosomal recessive disease with chronic and lethal evolution, predominantly present in the Caucasian populations. It is caused by mutations of the CF gene that encodes the action of the transmembrane cystic fibrosis conductance regulator (CFTR) protein, a Cl^- channel present in the cells that produces mucus, digestive enzymes, sweat, saliva and tears. The most important function of the CFTR protein is to ensure the transport of chloride ions in and out of the cells, thus contributing to the control of water circulation in tissues and ensuring the maintenance of the rheological properties of the mucus. The CFTR protein also has other complex functions; for example, it regulates the function of different channels, such as the sodium channel, thus ensuring sodium transport at the level of cell membranes and good functionality of the lungs and digestive system. CFTR is most frequently found in the apical membranes of epithelial cells secreting mucus and other proteins in different organs [1].

The physiological consequence of mucus dehydration is the appearance of viscous secretions adherent to the epithelia at the level of the mucous glands, which, over time, lead to the destruction of the respective organ. Thus, altered CFTR in the apical membrane of cholangiocytes will lead to biliary secretory alteration and the appearance of chronic cholangiopathy. Therefore, CF-associated liver disease (CFLD) has classically been considered a genetic channelopathy [2, 3]. However, only a fraction of CFTR patients develop CFLD. In addition, the clinical manifestations and severity of CFLD are different. Recent discoveries have shown that in the pathogenesis of CFLD, in addition to the dysfunction of cholangiocytes and the alteration of bile secretion, the CFTR phenotype and the impairment of epithelial innate immunity also contribute [3].

CFLD can present clinically in different ways, from moderate hepatomegaly, persistent increase in liver enzymes, micro-gallbladder, variceal haemorrhage and up to focal biliary cirrhosis and pulmonary hypertension [4]. According to the latest data in the literature, the prevalence of CFLD is approximately 23% in the CF population and increases linearly with age. The highest incidence was in children under 10 years of age. Children older than 10 years who had normal liver function did not develop CFLD. Thus, research to identify the cause should focus on young children [4, 5].

Definitions of CFLD over time have used clinical signs as disease classification criteria (Colombo, Debray and Koh) in order to be able to initiate treatment, while the Flass criteria have made a phenotypic classification more useful in research and the assessment of natural history [6-9]. As a result, these differences have led to different reports of the prevalence and incidence of CFLD and have not prevented the standardisation of diagnosis and recommended therapy. Based on these findings, the European and North American Society for Gastroenterology, Hepatology and Paediatric Nutrition (ESPGHAN and NASPGHAN) met and reached a consensus that standardised the classification of hepato-biliary manifestations as follows so that it can be used both for clinical and research purposes. Thus, Cystic Fibrosis Hepato-Biliary Implication (CFHBI) is an updated term that includes the extended spectrum of liver involvement, both from a clinical, diagnostic and phenotypic point of view observed in persons with CF (PwCF) [10].

In 2024, the Cystic Fibrosis Foundation (CFF) published a new guideline for the diagnosis of hepatobiliary complications associated with CF. The new CFHBI and advanced CF liver disease (aCFLD) definitions were adopted. Thus, the aCFLD is diagnosed when one or more changes are present: nodular liver, fibrosis (F4 - advanced form), multilobular cirrhosis with/ without portal hypertension, or noncirrhotic portal hypertension [11]. Also, the CFHBI is identified when one or more changes occur: hepatomegaly, liver fibrosis (< F4), on elastography increased liver stiffness (< F4), hepatic steatosis, focal biliary cirrhosis, persistent cholestasis (> 3 - 6 months), transaminase and gamma-glutamyl transferase (GGT) values above the highest value of normal, liver imaging changes, hepatolithiasis, cholelithiasis and sclerosing cholangitis [11].

Liver disease associated with CF is the third cause of mortality, after lung diseases and post-transplant complications [3, 12]. In addition, CFLD is an insidious disease because clinical symptoms appear late, when the liver damage is very advanced, with irreversible histopathological lesions. Some studies claim that early liver histopathological changes are reversible and can be treated if discovered in a timely manner [13]. CFLD has a high prevalence, early onset and unpredictable evolution; it is an urgent clinical necessity to find a method for early diagnosis of patients at risk of developing CFLD [7].

This study aimed to identify three novel non-invasive protein biomarkers to enhance the early detection of liver lesions in cystic fibrosis, thereby contributing to the reduction of morbidity and mortality in these individuals.

Materials and Methods

Patients and study protocol

This was an observational cross-sectional study. A number of 36 paediatric patients aged <18 years with CF were enrolled between November 2023 and March 2024 from two hospitals in Bucharest, Romania: "Alessandrescu-Rusescu" National Institute for Mother and Child Health (Regional Centre for CF) and "Grigore Alexandrescu" Clinical Emergency Hospital for Children. The study was conducted according to the guidelines of the Declaration of Helsinki and approved

by the Ethics Committee of the "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania. All the parents/owners of included participants signed the Informed Consent Form, and the agreement of the General Data Protection Regulation.

Inclusion criteria: 18 patients with a definite diagnosis of CF were included in the Control group. Other 18 patients with CF and hepatic impairment (CFHBI or aCFLD) were included in the Case group. All included participants had a definite diagnosis of CF through the sweat test, which is the "golden standard" and confirmed through genetic testing, especially where the sweat test was inconclusive. In accordance with the new consensus guidelines of CFF, the patients included in the study were divided as follows: PwCF, CFHBI and aCFLD [11].

All patients who did not have a definite diagnosis of CF by the "sweat test" and/or genetic confirmation and patients with other diseases that can cause liver damage not associated with CF were excluded. The opt-out criteria were as follows: any patient or their relative was free to withdraw from the study at any time before the end of the procedure.

Laboratory analyses

Serum protein analysis. We followed the recommended protocols for blood sampling. From each participant, a single peripheral blood sample (4 mL) was collected by venipuncture in a simple vacutainer without anti-coagulant. All samples were made from serum and prepared the same way after collection, according to the laboratory protocol: 30 minutes rest after collection, centrifugation at 2000 revolutions *per* minute, for 10 minutes. After obtaining the serum, the samples were divided into volumes of 300, 400 or 500 µL in microcentrifuge tubes with a volume of 1.5 mL, labelled and transported on ice for a maximum of one hour to a freezer at -80ºC, where they were kept. After finishing the collection from 36 subjects, the samples were transported on dry ice from Bucharest to Cluj-Napoca, Romania, at the MEDFUTURE Research Centre for Advanced Medicine. Serum levels of Human Ubiquitin Carboxyl-Terminal Hydrolase L1 (UCHL1), Endoglin/CD105 and Tissue Inhibitor of Metallo-proteinases-4 (TIMP-4) were quantified using sandwich ELISA kits. Each sample was measured in duplicate according to the manufacturer's instructions. To determine the levels of UCHL1, we used this kit: ABclonal, WoburnMA, USA, catalogue number RK09204, detection range = 78 - 50000 pg/mL sensitivity = 34 pg/mL, intra-assay precision coefficient of variation (CV) ≤ 5.1%, inter-assay precision CV ≤ 6.9%. For the determination of Endoglin, we used the same type of kit: ABclonal, WoburnMA, USA, catalogue number RK00275, detection range = 0.78 - 50 ng/mL, sensitivity = 39 pg/mL, intra-assay precision coefficient of variation (CV) ≤ 9.4%, inter-assay precision CV ≤ 9.1%. Also, the same type of kit was used for the determination of TIMP 4: ABclonal, WoburnMA, USA, catalogue number RK00147, detection range = 0.156 - 10 ng/mL sensitivity = 0.1 ng/mL, intra-assay precision coefficient of variation (CV) ≤ 10%, inter-assay precision CV ≤ 15%. For each parameter, a calibration curve was established using the protein standard provided with the kit. Absorbance was measured using a microplate reader (ClarioStar, BMGLabtech, Ortenberg, Germany), and data acquisition and analysis were conducted with the integrated Mars software. A 4-parameter logistic regression model was used to construct the calibration curve for quantification. The final concentration was obtained by averaging the two measurements.

Hepatic cytolysis samples and liver fibrosis markers. The other biomarkers like aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and platelet count (PLT) were measured in each of the two hospitals, according to standard procedures. All were performed during the period of clinical stability of the patients. Liver fibrosis markers were assessed using universally recognised formulas. The AST/ALT ratio was calculated as a simple division of AST by ALT levels. The GPR Score was determined using the formula:

$$\text{GPR Score} = [(\text{GGT}/\text{ULN} \times \text{GGT})/\text{PLT}] \times 100,$$

where, ULN GGT is the upper limit of normal for GGT.

The APRI Score was computed as:

$$\text{APRI Score} = [(\text{AST}/\text{ULN} \times \text{AST})/\text{PLT}] \times 100,$$

with ULN AST indicating the upper limit of normal for AST.

The FIB-4 Score was calculated using the equation [11]:

$$\text{FIB-4 Score} = (\text{Age in years} \times \text{AST})/(\text{PLT} \times \sqrt{\text{ALT}}).$$

To avoid bias (errors), we were very rigorous in calculating the APRI and GPR scores and used ULN for AST and GGT from each separate laboratory (from the 2 hospitals) and by age group. The division of patients into the 3 categories (PwCF and CFHBI or aCFLD) was made taking into account the increases in transaminases > 1.5 - 2 times compared to ULN and/or GGT, the changes in the liver ultrasound and the considered pathological values advanced liver fibrosis score (F4). The GPR score, with values ranging from 0.2 to 0.32, predicted moderate hepatic fibrosis, while a GPR score exceeding 0.6 indicated significant advanced fibrosis. Furthermore, a GPR score greater than 0.68, in conjunction with multiple liver ultrasound changes, suggested an increased risk of developing advanced cystic fibrosis liver disease (aCFLD). The APRI score of 0.5 or higher was predictive of significant fibrosis, whereas a score of 1 or greater indicated a likelihood of cirrhosis. Lastly, the FIB-4 score of 0.18 or above was associated with severe liver disease, and a score of 0.36 or higher was indicative of portal hypertension. In this study, we

considered several pathological values associated with the scores previously mentioned. The AST/ALT ratio was deemed pathological if it was equal to or greater than 1 [14]. The GPR Score, with values ranging from 0.2 to 0.32, predicted moderate hepatic fibrosis, while a GPR Score exceeding 0.6 predicted significantly advanced fibrosis [15, 16]. Furthermore, a GPR Score greater than 0.68, in conjunction with multiple liver ultrasound changes, suggested an increased risk of developing aCFLD [17]. An APRI score of 0.5 or higher was predictive of significant fibrosis, whereas a score of 1 or greater indicated a likelihood of cirrhosis [16, 18]. Lastly, the FIB-4 score of 0.18 or above was associated with severe liver disease, and a score of 0.36 or higher was indicative of portal hypertension [19].

Hepatic ultrasonography and transient elastography

All patients included in the study were evaluated by abdominal ultrasound. Evaluation of liver stiffness by transient elastography (TE) was done only in patients who showed ultrasound-increased liver size, hyperechoic echostructure, inhomogeneous, liver nodularity, irregular "crested" margins with nodular and micronodular appearance, periportal fibrosis and/or oesophageal varices and/or portal hypertension. Two patients required TE, those with aCFLD.

Statistical analysis

Statistical analyses were performed using XL-STAT version 2023.5 (Addinsoft, Paris, France) and VassarStats version SCR-010263 (Vassar College, New York, USA). Continuous variables were expressed as means with standard deviations (SDs), while categorical data were presented as frequencies and percentages. The two-tailed Fisher exact probability test was used to assess the relationships between categorical variables. At the same time, a one-way analysis of variance (ANOVA) was conducted to compare the median values of continuous variables between Case and Control groups. A p-value of < 0.05 was considered statistically significant. To assess the use specificity and sensibility of the analysed biomarkers, a receiver operating characteristic (ROC) curve was performed, and the area under the curve (AUC) was reported.

Results and Discussion

General Characteristics

The study included 36 patients, 18 in the Case and 18 in the Control groups. Patients included in the Case group included 16 patients with CFHBI and 2 patients with aCFLD. The general characteristics of the cohorts, along with the differences between them, are provided in Table I. Notably, the only characteristics that demonstrated a statistically significant difference were the median APRI score ($p = 0.008$), median FIB-4 score ($p = 0.03$) and the administration of ursodeoxycholic acid (UDCA) treatment ($p < 0.001$).

The analysis of genotype prevalence among Case and Control groups showed that the frequencies were similar ($p = 0.34$), and no genotype was associated with a higher risk of developing liver disease (Table II).

Table I

General characteristics of the patients

Parameter	Total cohort (n = 36)	Control group (PwCF; n = 18)	Case group (CFHBI or aCFLD; n = 18)	p-value
Median age, years (SD)	9.5 (4.67)	7.5 (4.92)	10 (4.46)	0.57
Males, n (%)	17 (47.2)	9 (50)	8 (44.4)	0.99
Urban areas, n (%)	16 (44.4)	9 (50)	7 (38.9)	0.74
Meconial ileus, n (%)	4 (11.1)	1 (5.56)	3 (16.7)	0.60
Pancreatic impairment, n (%)	36 (100)	18 (100)	18 (100)	1
Diabetes mellitus, n (%)	2 (5.56)	2 (11.1)	0 (0)	0.49
Hepatomegaly, n (%)	2 (5.56)	0 (0)	2 (11.1)	0.49
Splenomegaly, n (%)	0 (0)	0 (0)	0 (0)	1
AST/ALT >1, n (%)	18 (50)	9 (50)	9 (50)	1
Median AST/ALT (SD)	1.01 (0.84)	1.02 (0.39)	1.01 (1.13)	0.37
Median GPR Score (SD)	0.15 (0.52)	0.11 (0.09)	0.22 (0.66)	0.14
Median APRI score (SD)	0.24 (0.13)	0.22 (0.08)	0.29 (0.15)	**0.008**
Median FIB-4 score (SD)	0.15 (0.14)	0.13 (0.07)	0.19 (0.17)	**0.03**
Severe genotype, n (%)	26 (72.2)	11 (61.1)	13 (72.2)	0.51
UDCA treatment, n (%)	12 (33.3)	0 (0)	12 (66.7)	**< 0.001**
Modulator CFTR treatment, n (%)	23 (63.9)	12 (66.7)	11 (61.1)	0.99

Table II

The comparison of genotypes between Case and Control groups

Genotype	Total cohort (n = 36)	Control group (PwCF; n = 18)	Case group (CFHBI or aCFLD; n = 18)	p-value
DF508/DF508, n (%),	22 (61.1)	13 (72.2)	9 (50)	0.34
DF508/Other, n (%)	10 (27.8)	4 (22.2)	6 (33.3)	
Other/Other, n (%)	4 (11.1)	1 (5.6)	3 (16.7)	

TIMP-4 analyses

TIMP-4 was detectable in 12 patients (66.7%) in the Case group, respectively in 6 patients (33.3%) in the Control group. Therefore, the risk of having CFLD was twice higher in patients with laboratory-detectable levels of TIMP-4 (RR = 2, 95% CI: 0.96 - 4.15, p = 0.045).

The median value of TIMP-4 was 0.14 ng/mL (IQR: 0.12 - 0.16) in the Case group, respectively 0.09 ng/mL (IQR: 0.09 - 0.10) in the Control group. The median value of TIMP-4 was significantly higher in the Case group (p = 0.034; Figure 1). Notably, two patients with CFLD outlined the IQR of TIMP-4 values, one of them with 0.337 ng/mL and the other with 0.265 ng/mL.

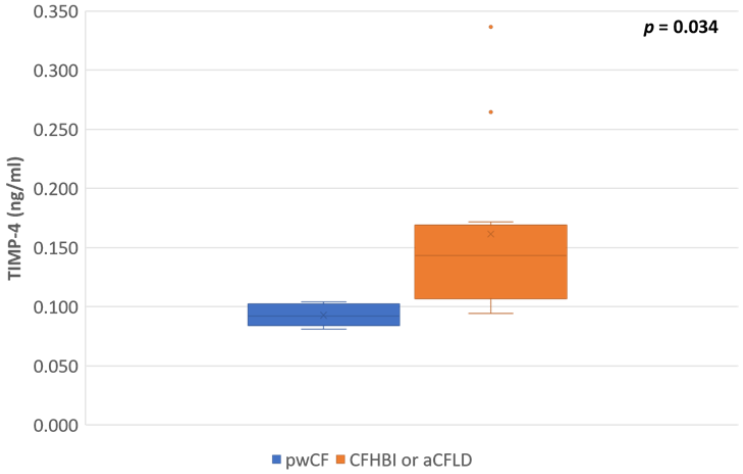

Figure 1.
The median values of TIMP-4 between patients with and without CFLD

The ROC-curve analysis showed that TIMP-4 had a high sensibility and specificity for detection of CFLD (AUC = 0.91, Figure 2). Specifically, for a cut-off value set at 0.1 ng/mL, the sensibility was 83% and the specificity was 67%.

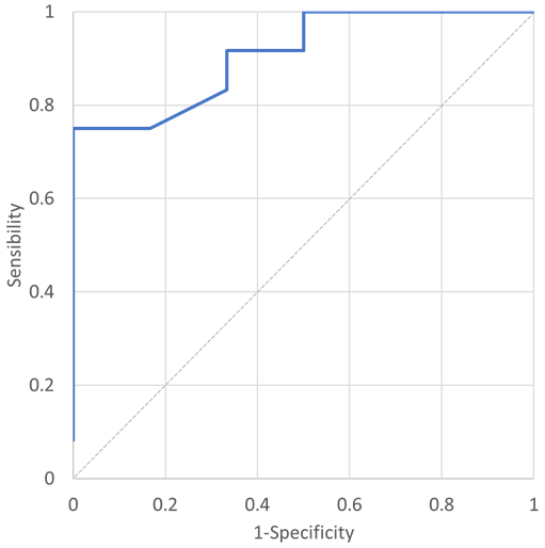

Figure 2.
The ROC curve of TIMP-4 between patients with and without CFLD

Endoglin analyses

The median value of Endoglin was 16.42 µg/mL (IQR: 16.19 - 16.61) in the Case group, respectively 16.25 µg/mL (IQR: 16.09 - 16.61) in the Control group. The median value of Endoglin was similar for both Case and Control groups (p = 0.26, Figure 3). Notably, three patients without CFLD outlined the IQR of Endoglin values, two with 15.42 µg/mL and the other with 15.35 µg/mL.

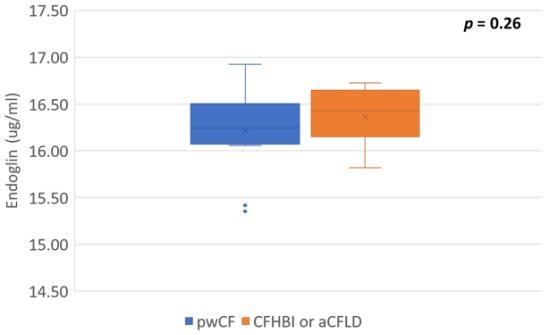

Figure 3.
The median values of Endoglin between patients with and without CFLD

The ROC-curve analysis showed that Endoglin had a low sensibility and specificity for the detection of CFLD (AUC = 0.58, Figure 4). Specifically, for a cut-off value set at 16 µg/mL, the sensibility was 89%, but the specificity was very low (17%).

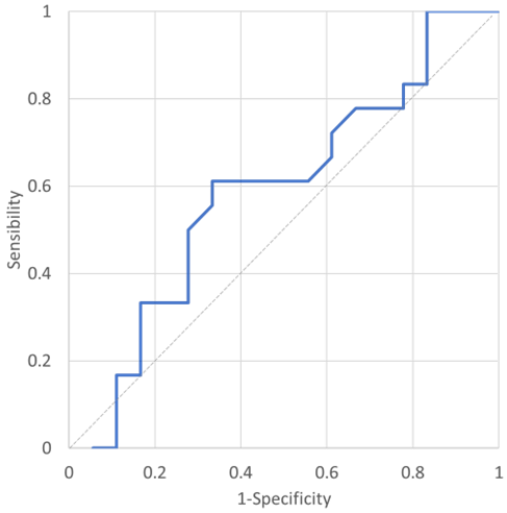

Figure 4.
The ROC curve of Endoglin between patients with and without CFLD

UCHL-1 analyses

The median value of UCHL-1 was 23.54 ng/mL (IQR: 15.76 - 29.72) in the Case group, respectively 23.54 ng/mL (IQR: 14.74 - 49.87) in the Control group. The median value of UCHL-1 was similar for both Case and Control groups (p = 0.55 Figure 5). Notably, one patient without CFLD outlined the IQR of UCHL-1 values, having 110.89 ng/mL.

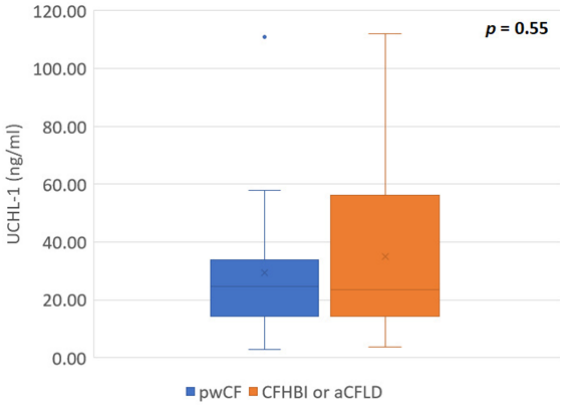

Figure 5.
The median values of UCHL-1 between patients with and without CFLD

The ROC-curve analysis showed that UCHL-1 had a low sensibility and specificity for detecting CFLD (AUC = 0.53, Figure 6). Specifically, for a cut-off value set at 20 ng/mL, the sensibility was 61% and the specificity was 49%.

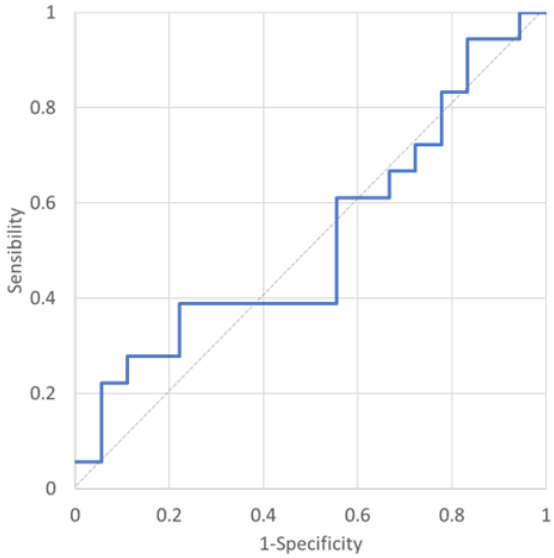

Figure 6.
The ROC curve of UCHL-1 between patients with and without CFLD

Selection of patients, liver fibrosis scores and CFTR genotype

Patient selection in this study was guided by the latest recommendations from the CFF. Our objective was to enhance the diagnostic accuracy for early liver damage detection, aiming to identify liver lesions at their initial onset stages. This approach was intended to improve the rate of early diagnosis and facilitate timely intervention [11]. The adoption of the CFHIB and aCFLD classifications, as opposed to the broader CFLD category, resulted in the inclusion of 16 patients with CFHIB and 2 with aCFLD in our study. To our knowledge, this is the first study employing this specific classification approach. This methodology enabled us to detect liver and biological changes well before irreversible liver damage could occur. According to the specialised literature, early detection is crucial as histopathological changes are potentially reversible only when identified at an early stage [13]. Identifying new biomarkers is crucial to reduce the reliance on liver biopsy, which, despite being considered the "gold standard" for diagnosing liver disease, is increasingly debated regarding its recommendation for focal liver disease in cystic fibrosis [7]. Additionally, liver biopsy is associated with significant intra- and inter-observer variability [20-22]. The literature highlights that liver biopsy is particularly challenging to accept among children and their parents, with repeated procedures being even less feasible due to its invasive nature [23-25].

In our study, the average age of patients with liver damage was 10 years, consistent with existing literature indicating that cystic fibrosis liver disease (CFLD) typically manifests within the first decade of life [26].

The APRI score, a simple and non-invasive biomarker, was developed to monitor liver pathology progression

and has been validated in clinical trials for accurately predicting liver fibrosis and cirrhosis [27]. It has been applied in studies involving children with hepatitis B, C and biliary atresia [28-31]. The FIB-4 index, derived from the APRICOT database, has been used in adults with various fibrotic liver diseases, including hepatitis C and non-alcoholic fatty liver disease [32].

In 2015, the first study involving paediatric patients with CFLD validated APRI and FIB-4 scores through liver biopsy, demonstrating that the APRI score was more effective than the FIB-4 score in differentiating CFLD from CF patients without liver disease (CFnoLD). The APRI score showed high specificity in predicting severe liver fibrosis but had limitations in identifying early-stage fibrosis [19]. A more recent study indicated a strong correlation between transient elastography (TE) values and the APRI score in CFLD patients, suggesting that these methods could be complementary for diagnosis and monitoring [33].

Our study found that APRI and FIB-4 scores were significantly elevated in patients with liver damage. Notably, the APRI score showed a significantly higher increase compared to the FIB-4 score in these patients, aligning with the literature that recognises the APRI score as the more reliable indicator of liver fibrosis [19, 33].

Regarding CFTR genotypes, our study did not find an association between severe lung disease genotypes – the DF508/DF508 homozygous and the DF508/G542X heterozygous – and an increased risk of liver disease, contrary to some previous reports. All patients in our study had pancreatic insufficiency, which was consistent with the presence of severe disease phenotypes as described in the literature [34].

Evaluating TIMP-4 and Endoglin as non-invasive biomarkers

Few studies have explored proteomic biomarkers in cystic fibrosis, particularly in paediatric populations. Recent research by Rath *et al.* demonstrated that both TIMP-4 and endoglin levels are elevated in patients with CFLD and correlate with liver damage staging. Their findings suggest that these biomarkers, when used in conjunction with TE, provide enhanced sensitivity for the non-invasive diagnosis of CFLD, including in children. Elevated endoglin levels were notably associated with the severity of liver damage, indicating its potential role in liver fibrosis pathology [35].

In our study, TIMP-4 levels were significantly higher in the Case group, corroborating previous findings [35]. Notably, two outliers had TIMP-4 levels substantially above the average: one with a value of 0.337 ng/mL, who had aCFLD (cirrhosis grade I) and the highest GPR score (2.74) and another with a value of 0.265 ng/mL, who had moderate fibrosis and a lower GPR score (0.23). Both outliers were undergoing treatment with CFTR modulators for 18 months. This raises the question of whether TIMP-4 could be a useful biomarker for monitoring liver fibrosis progression during CFTR modulator therapy. Given its non-invasive nature and early presence in liver fibrosis, TIMP-4 shows promise as a future biomarker in cystic fibrosis management.

Conversely, in a study of children with biliary atresia but without cystic fibrosis, endoglin levels were significantly correlated with increased liver stiffness measurement (LSM), suggesting its potential utility in predicting liver stiffness [35, 36].

However, our study did not find a significant difference in endoglin levels between the Case and Control groups, indicating that it may not be a suitable biomarker for evaluating liver fibrosis in cystic fibrosis patients.

Evaluating UCHL-1 as a biomarker

UCHL-1 is less well-studied compared to other biomarkers, but emerging research highlights its potential role in liver disease. Wilson *et al*. demonstrated an association between UCHL-1 levels and the activation of hepatic stellate cells (HSC), suggesting its involvement in regulating HSC proliferation and its potential as a pharmacological target in chronic liver disease [37]. In another study, UCHL-1 was found to be elevated in the plasma of patients with chronic hepatitis C, with a plasma value greater than 0.39 ng/mL considered positive [38]. This increase was attributed to UCHL-1's role as a deubiquitinase, which is significantly elevated following HSC activation [38]. Lewindon *et al.* further noted that HSCs are crucial in the fibrogenesis associated with CFLD due to their cytokine production in the bile duct epithelium [39].

Only one experimental study has previously linked UCHL-1 to cystic fibrosis, finding that UCHL-1 protects CFTR from proteasomal degradation during early misfolding stages [40].

In our study, we hypothesised that UCHL-1 might offer protective effects on HSCs and/or cholangiocyte epithelium in liver fibrosis. However, our results showed no significant differences in UCHL-1 levels between the two groups. The elevated UCHL-1 values observed in both groups could indicate early activation of HSCs in cystic fibrosis, potentially occurring before other clinical or biological signs become apparent.

Our study primarily focused on the roles of TIMP-4, UCHL-1 and Endoglin in CFLD. However, these biomarkers have broader implications across various liver diseases, which increases the relevance of our findings.

Endoglin, as a co-receptor for transforming growth factor-beta (TGF-β), plays a pivotal role in liver fibrogenesis [35, 39], and is linked to increased liver stiffness in conditions like biliary atresia and hepatitis, making it a potential biomarker for fibrosis severity [36]. While our study did not find significant differences in endoglin levels in CFLD, this may reflect the distinct pathophysiology of CFLD compared to other liver diseases. Future research should investigate how

endoglin varies with fibrosis progression or therapeutic interventions, including CFTR modulators.

UCHL-1 is increasingly recognised for its role in liver fibrosis, particularly through hepatic stellate cell (HSC) activation [37]. Elevated UCHL-1 levels in chronic hepatitis C have been linked to advanced fibrosis, suggesting its potential as a biomarker for early fibrogenic activity [38]. Its role in proteasomal regulation and cellular stress responses may also have implications for liver repair [40]. The elevated UCHL-1 levels observed in both groups in our study might indicate early HSC activation, which is common in chronic liver conditions. However, its specific role in CFLD needs further clarification. These findings highlight the importance of proteomic biomarkers in liver disease assessment. While TIMP-4 shows promise for early diagnosis and monitoring, exploring Endoglin and UCHL-1 in other liver diseases, such as biliary atresia or chronic hepatitis C, could broaden their clinical utility. Longitudinal studies focusing on their responsiveness to therapies (*e.g.*, CFTR modulators or antifibrotic agents) are needed to confirm their role in dynamic disease monitoring.

The role of TIMP-4 in CFLD fibrosis is closely tied to its regulation of extracellular matrix (ECM) turnover through matrix metalloproteinases (MMP) inhibition, preventing ECM degradation and promoting fibrogenesis [35]. This aligns with endoglin's function in TGF-β signalling, which enhances HSC activation and ECM deposition, suggesting a cooperative mechanism in the CFLD fibrosis [36, 39]. Similarly, UCHL-1, a deubiquitinase involved in HSC activation and ECM remodelling [38], may interact with TIMP-4 within broader fibrotic networks. Elevated TIMP-4 levels, particularly in patients on CFTR modulator therapy, indicate its potential as a biomarker for fibrosis progression and therapeutic response. Further exploration of the interplay between TIMP-4, endoglin and UCHL-1 in CFLD could yield valuable insights into the mechanisms driving liver fibrosis. Specifically, longitudinal studies examining how TIMP-4 levels correlate with changes in endoglin or UCHL-1 during fibrosis progression or treatment may help clarify their roles in fibrotic pathways. Experimental models focusing on HSC activation, TGF-β signalling and ECM turnover could also elucidate the molecular connections between these biomarkers, enhancing their utility for diagnostic and therapeutic applications in CFLD.

Our study has several notable strengths. It highlighted the potential of TIMP-4 as a proteomic biomarker for the early detection of reversible liver changes in CF, with its value correlating with established liver fibrosis scores (APRI and FIB-4). This research represents the first multicentre study in Romania focusing exclusively on paediatric patients to identify proteomic biomarkers for the early diagnosis of CFLD. Additionally, it is the first to assess UCHL-1 levels in the serum of CF patients and to apply the latest CFF guidelines and recommendations for patient inclusion [11]. Notably, our study also poses the novel question of whether TIMP-4 could serve as a safe, cost-effective and non-invasive biomarker for monitoring liver fibrosis progression during treatment with CFTR modulators, especially given that transaminase levels are not always indicative of liver changes. Despite these strengths, the study faced limitations, particularly regarding the inconsistent diagnostic criteria for liver damage in CF across the participating centres.

Conclusions

Our study demonstrates a significant association between TIMP-4 and the presence of early liver fibrosis lesions in patients with cystic fibrosis. This association is further supported by its correlation with established fibrosis indices, such as APRI and FIB-4 scores. Notably, a TIMP-4 serum concentration exceeding 0.30 ng/mL emerged as a potential threshold for suspecting the onset of CFLD. Consequently, TIMP-4 shows promise as an early diagnostic marker for CFHBI. Its detection in a child's serum could serve as an initial indicator of possible CFLD development. Given that TIMP-4 measurement is non-invasive, cost-effective and rapid, it holds significant potential for clinical application in early detection of liver damage in CF.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Fiorotto R, Strazzabosco M, Cystic Fibrosis–Related Liver Diseases: New Paradigm for Treatment Based on Pathophysiology. *Clin Liver Dis.*, 2016; 8(5): 113-116.
2. Fiorotto R, Strazzabosco M, Pathophysiology of Cystic Fibrosis Liver Disease: A Channelopathy Leading to Alterations in Innate Immunity and in Microbiota. *Cell Mol Gastroenterol Hepatol.*, 2019; 8(2): 197-207.
3. Valamparampil JJ, Gupte GL, Cystic fibrosis associated liver disease in children. *World J Hepatol.*, 2021; 13(11): 1727-1742.
4. Wasuwanich P, Karnsakul W, Cystic fibrosis-associated liver disease in children. *Minerva Pediatr.*, 2020; 72(5): 440-447.
5. Rowland M, Drummond J, Connolly L, Daly E, McCormick PA, Bourke B, Cystic Fibrosis Registry of Ireland, Cystic Fibrosis Liver Disease Research Study Group, The natural history of cystic fibrosis liver disease a prospective cohort study. *J Cyst Fibros.*, 2023; 22(6): 1054-1061.

6. Colombo C, Battezzati PM, Crosignani A, Morabito A, Costantini D, Padoan R, Giunta A, Liver disease in cystic fibrosis: A prospective study on incidence, risk factors, and outcome. *Hepatology*, 2002; 36(6): 1374-1382.
7. Debray D, Kelly D, Houwen R, Strandvik B, Colombo C, Best practice guidance for the diagnosis and management of cystic fibrosis-associated liver disease. *J Cyst Fibros.*, 2011; 10(SUPPL.2): S29-S36.
8. Koh C, Sakiani S, Surana P, Zhao X, Eccleston J, Kleiner DE, Herion D, Liang TJ, Hoofnagle JH, Chernick M, Heller T, Adult-onset cystic fibrosis liver disease: Diagnosis and characterization of an underappreciated entity. *Hepatology*, 2017; 66(2): 591-601.
9. Flass T, Narkewicz MR, Cirrhosis and other liver disease in cystic fibrosis. *J Cyst Fibros.*, 2013; 12(2): 116-124.
10. Bodewes FAJA, Freeman AJ, Weymann A, Debray D, Scheers I, Verkade HJ, Narkewicz MR, Towards a Standardized Classification of the Hepatobiliary Manifestations in Cystic Fibrosis (CFHBI): A Joint ESPGHAN/NASPGHAN Position Paper. *J Pediatr Gastroenterol Nutr.*, 2024; 78(1): 153-165.
11. Sellers ZM, Assis DN, Paranjape SM, Sathe M, Bodewes F, Bowen M, Cipolli M, Debray D, Green N, Hughan KS, Hunt WR, Leey J, Ling SC, Morelli G, Peckham D, Pettit RS, Philbrick A, Stoll J, Vavrina K, Allen S, Goodwin T, Hempstead SE, Narkewicz MR, Cystic fibrosis screening, evaluation, and management of hepatobiliary disease consensus recommendations. *Hepatology*, 2024; 79(5): 1220-1238.
12. Alexopoulou A, Pouriki S, Vasilieva L, Alexopoulos T, Filaditaki V, Gioka M, Diamantea F, Dourakis SP, Evaluation of noninvasive markers for the diagnosis of cystic fibrosis liver disease. *Scand J Gastroenterol.*, 2018; 53(12): 1547-1552.
13. Kobelska-Dubiel N, Klincewicz B, Cichy W, Liver disease in cystic fibrosis. *Prz Gastroenterol.*, 2014; 9(3): 136-141.
14. Lieber CS, Weiss DG, Paronetto F, Veterans Affairs Cooperative Study 391 Group, Value of fibrosis markers for staging liver fibrosis in patients with precirrhotic alcoholic liver disease. *Alcohol Clin Exp Res.*, 2008; 32(6): 1031-1039.
15. Calvopina DA, Lewindon PJ, Ramm LE, Noble C, Hartel GF, Leung DH, Ramm GA, Gamma-glutamyl transpeptidase-to-platelet ratio as a biomarker of liver disease and hepatic fibrosis severity in paediatric Cystic Fibrosis. *J Cyst Fibros.*, 2022; 21(2): 236-242.
16. Karnsakul W, Wasuwanich P, Ingviya T, Vasilescu A, Carson KA, Mogayzel PJ, Schwarz KB, A longitudinal assessment of non-invasive biomarkers to diagnose and predict cystic fibrosis-associated liver disease. *J Cyst Fibros.*, 2020; 19(4): 546-552.
17. Sellers ZM, Lee LW, Barth RA, Milla C, New Algorithm for the Integration of Ultrasound Into Cystic Fibrosis Liver Disease Screening. *J Pediatr Gastroenterol Nutr.*, 2019; 69(4): 404-410.
18. Shaheen AA, Myers RP, Diagnostic accuracy of the aspartate aminotransferase-to-platelet ratio index for the prediction of hepatitis C-related fibrosis: a systematic review. *Hepatology*, 2007; 46(3): 912-921.
19. Leung DH, Khan M, Minard CG, Guffey D, Ramm LE, Clouston AD, Miller G, Lewindon PJ, Shepherd RW, Ramm GA, Aspartate aminotransferase to platelet ratio and fibrosis-4 as biomarkers in biopsy-validated pediatric cystic fibrosis liver disease. *Hepatology*, 2015; 62(5): 1576-1583.
20. Colloredo G, Guido M, Sonzogni A, Leandro G, Impact of liver biopsy size on histological evaluation of chronic viral hepatitis: the smaller the sample, the milder the disease. *J Hepatol.*, 2003; 39(2): 239-244.
21. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, Feng ZZ, Reddy KR, Schiff ER, Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol.*, 2002; 97(10): 2614-2618.
22. Bedossa P, Dargère D, Paradis V, Sampling Variability of Liver Fibrosis in Chronic Hepatitis C. *Hepatology*, 2003; 38(6): 1449-1457.
23. Bravo AA, Sheth SG, Chopra S, Liver Biopsy. *N Engl J Med.*, 2001; 344(7): 495-500.
24. Cadranel JF, Rufat P, Degos F, Practices of liver biopsy in France: Results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology*, 2000; 32(3): 477-481.
25. Castera L, Negre I, Samii K, Buffet C, Pain experienced during percutaneous liver biopsy. *Hepatology*, 1999; 30(6): 1529-1530.
26. Parisi GF, Di Dio G, Franzonello C, Gennaro A, Rotolo N, Lionetti E, Leonardi S, Liver disease in cystic fibrosis: an update. *Hepat Mon.*, 2013; 13(8): e11215.
27. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS, A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology*, 2003; 38(2): 518-526.
28. Jin W, Lin Z, Xin Y, Jiang X, Dong Q, Xuan S, Diagnostic accuracy of the aspartate aminotransferase-to-platelet ratio index for the prediction of hepatitis B-related fibrosis: a leading meta-analysis. *BMC Gastroenterol.*, 2012; 12: 14.
29. Lin ZH, Xin YN, Dong QJ, Wang Q, Jiang XJ, Zhan SH, Sun Y, Xuan SY, Performance of the aspartate aminotransferase-to-platelet ratio index for the staging of hepatitis C-related fibrosis: an updated meta-analysis. *Hepatology*, 2011; 53(3): 726-736.
30. McGoogan KE, Smith PB, Choi SS, Berman W, Jhaveri R, Performance of the AST-to-platelet ratio index as a noninvasive marker of fibrosis in pediatric patients with chronic viral hepatitis. *J Pediatr Gastroenterol Nutr.*, 2010; 50(3): 344-346.
31. Grieve A, Makin E, Davenport M, Aspartate Aminotransferase-to-Platelet Ratio index (APRi) in infants with biliary atresia: prognostic value at presentation. *J Pediatr Surg.*, 2013; 48(4): 789-795.
32. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, Sulkowski MS, Torriani FJ, Dieterich DT, Thomas DL, Messinger D, Nelson M, APRICOT Clinical Investigators, Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology*, 2006; 43(6): 1317-1325.

33. Woolfson JP, Schreiber RA, Raveendran S, Chilvers M, Barker C, Guttman OR, Role of transient elastography and APRI in the assessment of pediatric cystic fibrosis liver disease. *Can Liver J.*, 2021; 4(1): 23-32.
34. Wilschanski M, Rivlin J, Cohen S, Augarten A, Blau H, Aviram M, Bentur L, Springer C, Vila Y, Branski D, Kerem B, Kerem E, Clinical and genetic risk factors for cystic fibrosis-related liver disease. *Pediatrics*, 1999; 103(1): 52-57.
35. Rath T, Hage L, Kügler M, Menendez Menendez K, Zachoval R, Naehrlich L, Schulz R, Roderfeld M, Roeb E, Serum proteome profiling identifies novel and powerful markers of cystic fibrosis liver disease. *PLoS One*, 2013; 8(3): e58955.
36. Leung DH, Devaraj S, Goodrich NP, Chen X, Rajapakshe D, Ye W, Andreev V, Minard CG, Guffey D, Molleston JP, Bass LM, Karpen SJ, Kamath BM, Wang KS, Sundaram SS, Rosenthal P, McKiernan P, Loomes KM, Jensen MK, Horslen SP, Bezerra JA, Magee JC, Merion RM, Sokol RJ, Shneider BL, Alonso E, Bass L, Kelly S, Riordan M, Melin-Aldana H, Bezerra J, Bove K, Heubi J, Miethke A, Tiao G, Denlinger J, Chapman E, Sokol R, Feldman A, Mack C, Narkewicz M, Suchy F, Sundaram SS, Van Hove J, Garcia B, Kauma M, Kocher K, Steinbeiss M, Lovell M, Loomes KM, Piccoli D, Rand E, Russo P, Spinner N, Erlichman J, Stalford S, Pakstis D, King S, Squires R, Sindhi R, Venkat V, Bukauskas K, McKiernan P, Haberstroh L, Squires J, Rosenthal P, Bull L, Curry J, Langlois C, Kim G, Teckman J, Kociela V, Nagy R, Patel S, Cerkoski J, Molleston JP, Bozic M, Subbarao G, Klipsch A, Sawyers C, Cummings O, Horslen SP, Murray K, Hsu E, Cooper K, Young M, Finn L, Kamath BM, Ng V, Quammie C, Putra J, Sharma D, Parmar A, Guthery S, Jensen K, Rutherford A, Lowichik A, Book L, Meyers R, Hall T, Wang KS, Michail S, Thomas D, Goodhue C, Kohli R, Wang L, Soufi N, Thomas D, Karpen S, Gupta N, Romero R, Vos MB, Tory R, Berauer JP, Abramowsky C, McFall J, Shneider BL, Harpavat S, Hertel P, Leung D, Tessier M, Schady D, Cavallo L, Olvera D, Banks C, Tsai C, Thompson R, Doo E, Hoofnagle J, Sherker A, Torrance R, Hall S, Magee J, Merion R, Spino C, Ye W, Childhood Liver Disease Research Network, Serum biomarkers correlated with liver stiffness assessed in a multicenter study of pediatric cholestatic liver disease. *Hepatology*, 2023; 77(2): 530-545.
37. Wilson CL, Murphy LB, Leslie J, Kendrick S, French J, Fox CR, Sheerin NS, Fisher A, Robinson JH, Tiniakos DG, Gray DA, Oakley F, Mann DA, Ubiquitin C-terminal hydrolase 1: A novel functional marker for liver myofibroblasts and a therapeutic target in chronic liver disease. *J Hepatol.*, 2015; 63(6): 1421-1428.
38. Cheng JC, Tseng CP, Liao MH, Peng CY, Yu JS, Chuang PH, Huang JT, Chen JJW, Activation of hepatic stellate cells by the ubiquitin C-terminal hydrolase 1 protein secreted from hepatitis C virus-infected hepatocytes. *Sci Rep.*, 2017; 7(1): 4448.
39. Lewindon PJ, Pereira TN, Hoskins AC, Bridle KR, Williamson RM, Shepherd RW, Ramm GA, The role of hepatic stellate cells and transforming growth factor-beta(1) in cystic fibrosis liver disease. *Am J Pathol.*, 2002; 160(5): 1705-1715.
40. Henderson MJ, Vij N, Zeitlin PL, Ubiquitin C-terminal hydrolase-L1 protects cystic fibrosis transmembrane conductance regulator from early stages of proteasomal degradation. *J Biol Chem.*, 2010; 285(15): 11314-11325.