Original paper

The use of transferrin isoforms to differentiate cholestatic liver diseases

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Abstract

Introduction: There are two main chronic autoimmune cholestatic liver diseases, primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), which may lead to liver fibrosis or cirrhosis and consequent disturbances in protein and glycoprotein synthesis and glycosylation. One of the glycoproteins synthesised in the liver is transferrin (TRF).
Aim of the research: The aim of the study was to compare the serum profile of TRF isoforms in autoimmune cholestatic liver diseases and extrahepatic cholestasis.
Material and methods: The study groups consisted of 76 patients with PBC, 100 patients with PSC, and 40 patients with extrahepatic cholestasis. Capillary electrophoresis was used to determine the profile of TRF isoforms.
Results: The differences in the TRF glycosylation occur in PBC and extrahepatic cholestasis. The mean concentrations of di- and trisialotransferrin were significantly lower in PBC patients than those in the controls. Additionally, the concentration of disialotransferrin differed significantly between the PBC and PSC groups. The area under the ROC curve for disialotransferrin in PBC patients (AUC = 0.819) was significantly higher than for PSC patients (AUC = 0.572). In turn, the mean concentrations of pentasialotransferrin in PBC and PSC were higher, and the mean concentrations of tetra- and disialotransferrin were lower in autoimmune cholestatic liver diseases than in extrahepatic cholestasis. None of the transferrin isoforms showed differences depending on the stage of liver fibrosis.
Conclusions: These differences enable the use of TRF isoforms in the differential diagnosis between these diseases.

Introduction

Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are chronic autoimmune biliary diseases with specific clinical symptoms and characteristics [1, 2]. The World Health Organisation (WHO) has allocated them separate codes [3]. The diagnosis of PSC requires the increased activity of cholestatic liver enzymes (alkaline phosphatase in particular) and diagnostic cholangiography performed by means of endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiography (MRCP), or percutaneous transhepatic cholangiography [4]. Antineutrophil nuclear antibodies (ANNA) are serological markers that can be present in 30% to 80% of cases [4]. The diagnosis of PBC is based on the confirmation of cholestasis, serologic reactivity to antimitochondrial antibodies (AMA), or specific antinuclear antibody (ANA) reactivity, with accompanying histologic evidence of chronic non-suppurative, granulomatous, lymphocytic small bile duct cholangitis [5, 6]. Raised serum alkaline phosphatase activity is characteristic of a typical cholestatic biochemical profile in both diseases [7, 8], and the both diseases are often progressive, resulting in end-stage liver disease [4, 5].

Aim of the research

In this work, we propose to study transferrin isoforms in autoimmune cholestatic liver diseases and extrahepatic cholestasis, as a marker of protein glycosylation changes, which can be a helpful tool for laboratory differentiation of cholestatic liver disease of different aetiologies. Previously, the changes in the profile of transferrin isoforms in PBC [9] and other liver diseases [10, 11] have been detected.

Material and methods

Patients

The study was conducted on the sera of 76 patients with primary biliary cholangitis (68 women and 8 men: median age for women: 53 years, range: 22–75 years; median age for men: 46 years, range: 34–57 years) and 100 patients with primary sclerosing cholangitis (79 men, 21 women: median age for men: 38 years; range 26–74; median age for women: 38 years; range: 26–75 years) treated in the Department of Gastroenterology, Hepatology, and Clinical Oncology of the Centre of Postgraduate Medical Education, Warsaw, Poland. A group

Table 1. Characteristics of healthy and sick groups

Parameter	Healthy (*N* = 40)	PBC (*N* = 76)	PSC (*N* = 100)	ECh (*N* = 40)
Age (range)	35 (20–60)	52 (22–75)	38 (26–75)	48 (32–64)
Sex (F/M)	30/10	68/8	21/79	17/23
AST [UI/l]	23 14–39	240 84–4374	135*# 30–453	66.5*δ§ 20–729
ALT [UI/l]	17.5 6.0–39.0	150* 36–4002	108*# 525–754	36*δ§ 11–667
ALP [IU/l]	58 31–92	208.5* 38.4–1171	184* 39–691	366*δ§ 107–855
GGT [IU/l]	23.5 8–39	450* 48–2058.6	232.5*# 12–912	242*δ 16–2226
Bilirubin [µmol/l]	13.3 4.3–21.0	17.96* 4.27–649	13.60# 2.56–49.25	29.7*δ§ 5.47–649
Transferrin [g/l]	2.89 1.84–4.20	3.26* 0.39–6.70	3.26 1.55–6.70	2.4*δ§ 0.95–4.38
BAs [µM/l]	3.80 1.11–9.80	28.5* 14.5–75	39.35*# 13.60–95.10	–
AMA-M2 (+/–) (%)	0/76 (0)	64/76 (84.2)	0/100 (0.0)	–
Anti-gp210 (+/–) (%)	0/76 (0)	26/76 (34.2)	0/100 (0.0)	–
Anti-SP100 (+/–) (%)	0/76 (0)	27/76 (35.5)	3/100 (3.0)	–
Anti-Ro52 (+/–) (%)	0/76 (0	26/76 (34.2)	–	–
Anti-p62 (+/–) (%)	0/76 (0)	4/76 (5.2)	5/100 (5.0)	–
Anti-centromere (+/–) (%)	0/76 (0)	6/76 (7.9)	–	–
Anti-PR3 IgG (+/-) (%)	–	–	33/100 (33)	–

*Data are median and ranges. ECh – extrahepatic cholestasis, *statistically significant difference between tested groups and controls, #statistically significant difference between PBC and PSC, δstatistically significant difference between ECh and PBC, §statistically significant difference between ECh and PSC.*

of 40 patients with extrahepatic cholestasis (carcinoma of Vater's ampulla) was also collected. The sera of 40 healthy subjects were assayed as a control group. All patients gave their informed consent to participate in the study. The tests were performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the Medical University in Bialystok (Protocol number R-I-002/563/2019 for PBC study and APK.002.232.2021 for PSC study). The characteristics of the patients and healthy controls are presented in Table 1.

The occurrence of autoantibodies in the PBC patients was as follows: anti-Sp100: 35.53% (27/76), anti-gp210 and anti-Ro52: 34.21%, anti-centromere: 7.89%, anti-p61: 5.26%, and AMAM2: 84.21%; and in PSC patients, anti-PR3 IgG: 33%, anti-Sp: 3%, and anti-p62: 5%.

Diagnosis

The diagnosis of PBC was made on the basis of the guidelines of the European Association for the Study of the Liver (EASL) [7] and PSC according to the American Collage of Gastroenterology Clinical Guidelines [8]. The patients had not been treated before their diagnosis or before examinations were conducted. The main criteria for PBC diagnosis included elevated alkaline phosphatase activity, the presence of AMA and/or anti-2-oxoacid dehydrogenase complex and/or PBC-specific ANA, cholangitis in histopathologic testing, and the destruction of small- or medium-sized bile ducts. Differentiation from PSC was made on the basis of liver biopsy. The diagnosis of PSC was made on the basis of MRCP or ERCP, changes in the activity of cholestatic enzymes (ALP and GGT), and after the exclusion of other causes of inflammation or strictures of bile ducts. The staging of liver fibrosis in PBC patients was performed according to Ludwig's score (15.2% of patients – no fibrosis, 48.5% of patients – mild fibrosis, 22.7% of patients – moderate fibrosis, and 13.6% of patients – severe fibrosis), and in PSC patients according to FibroTest (36.7% of patients – no fibrosis, 35% of patients – insignificant fibrosis [scores F0-F1, F1, F1-F2], and 28.3% of patients – significant fibrosis [scores F2, F3, F3-F4, F4]). Thus, significant fibrosis occurred in 36.3% (22.7% + 13.6%) of PBC patients and in 28.3% of PSC patients. Fibrosis

of varying degrees was present in 84.8% of patients with PBC and in 63.3% of patients with PSC, i.e. no fibrosis was observed twice as often in patients with PSC (36.7%) as in patients with PBC (15.2%).

Autoantibodies assay

Autoantibodies were detected by means of commercially available ELISA kits: anti-Sp100 antibodies with an IMTEC Sp100 antibodies kit (ITC 660040; IMTEC, Berlin, Germany); anti-gp210 with a QUANTA Lite gp210 kit (Inova Diagnostics, San Diego, CA, USA); anti-AMA M2 using an IMTEC-AMA M2 kit (HUMAN-IMTEC, Berlin, Germany); anti-Ro-52 with a QANTA Lite SS-A 52 ELISA kit (Inova Diagnostics, San Diego, CA, USA); and anti-centromere using an ACA QANTA Lite Centromere ELISA kit (CENP-A & CENP-B) (Inova Diagnostics, San Diego, CA, USA). Anti-p62 antibodies were measured by the method outlined by Bauer and Habior [12]. Anti-PR-3 IgG was determined using the ELISA method with PR-3 ANCA test (Human Diagnostics, Wiesbaden, Germany) on an automatic plate reader (Multiscan RC, Labsystem, Vantaa, Finland).

Transferrin isoform assay

Transferrin isoforms were separated using the capillary electrophoresis (CE) method on the MINICAP electrophoretic system using the MINICAP CDT reagent kit (Sebia, Evry, France). Asialotransferrin, disialotransferrin, trisialotransferrin, tetrasialotransferrin, and pentasialotransferrin can be detected using this method. The concentration of each fraction was automatically calculated as the relative value (%) of total transferrin (relative concentration).

Total transferrin assay

The total serum concentration of transferrin was measured using the immunoturbidimetric method with a transferrin kit (TRFS Gen.2) from Roche (Roche Diagnostics International AG, Rotkreuz, Switzerland) on a Cobas c501 analyser (Roche Diagnostics, AG, Rotkreuz, Switzerland).

Total bile acid assay

Total bile acids were determined by the enzyme cycling method using the Diazyme total bile acids assay kit (Diazyme Laboratories, Gregg Court, Poway, USA) on an Indiko Plus analyser (Thermo Fisher Scientific, Thermo, Finland).

Statistical analysis

The results were presented as the median and ranges. The differences between the patients and the controls were evaluated using the Mann–Whitney *U* test. The GraphROC for Windows with Medical Set (version 13.3) was used to calculate the diagnostic accuracy and to compare the areas under ROC curves of the isoforms (2-sialo- and 3-sialo-transferrin) between the different diseases. The differences were considered statistically significant at $p < 0.05$.

Results

There were no differences in the concentration of transferrin isoforms depending on age and gender; therefore, we present the results for the entire study groups ($p > 0.05$ for all comparisons in Mann-Whitney *U*-test and in the test for Spearman's rank correlation coefficient).

None of the transferrin isoforms changed in patients with PSC in comparison to the control group, but the concentration of disialotransferrin (median: 0.30%; ranges: 0.00–0.90) and trisialotransferrin (median: 3.00%; ranges: 0.00–5.70) were significantly lower in patients with PBC compared with the control group (median: 0.60; range: 0.30–1.20 for disialotransferrin and median: 3.65; range: 1.60–5.10 for trisialotransferrin) ($Z = -4.279$; $p < 0.001$ and $Z = -2.395$; $p = 0.017$; respectively) (Figure 1).

The concentration of disialotransferrin in PBC patients was significantly lower than that in PSC patients (median: 0.60%; ranges: 0.20–1.30) ($Z = 3.748$; $p < 0.001$). It is clearly visible that the concentrations of di- and trisialotransferrin in PBC patients coincide with the concentrations of isoforms in PSC patients.

The concentration of pentasialotransferrin in extrahepatic cholestasis (ECh) patients was significantly lower and the concentration of tetrasialotransferrin significantly higher than that in the control group ($Z = -2.403$; $p = 0.016$ and $Z = 3.259$; $p = 0.001$, respectively). The concentrations of pentasialotransferrin in the PBC and PSC groups were significantly higher than that in the ECh group ($Z = 5.797$; $p < 0.001$ and $Z = 5.543$; $p < 0.001$, respectively), but the concentrations of tetrasialotransferrin in both groups were significantly lower than that in the ECh group ($Z = -4.780$; $p < 0.001$ and $Z = -5.500$; $p < 0.001$, respectively).

The area under the curve (AUC) for disialotransferrin in PBC patients (AUC = 0.819; confidence interval: 0.757–0.929) was significantly higher than the AUC in PSC patients (AUC = 0.572; confidence interval: 0.431–0.712) ($p < 0.001$) and in ECh (AUC = 0.517; confidence interval: 0.362–0.672) ($p < 0.001$) (Figure 2). AUC for trisialotransferrin in PBC (AUC = 0.675; confidence interval: 0.541–0.808) was higher than that in PSC (AUC = 0.590; confidence interval: 0.439–0.741) ($p = 0.038$) and in ECh (AUC = 0.638; confidence interval: 0.495–0.78) ($p = 0.013$). AUC for tetrasialotransferrin in ECh (AUC = 0.748; confidence interval: 0.611–0.885) was significantly higher than that in PBC (AUC = 0.516; confidence interval: 0.367–0.665) ($p < 0.001$) and in PSC (AUC = 0.517, confidence interval: 0.361–0.673) ($p < 0.001$). AUC for pentasialotransferrin in ECh (AUC = 0.683; confidence interval: 0.523–0.843) was

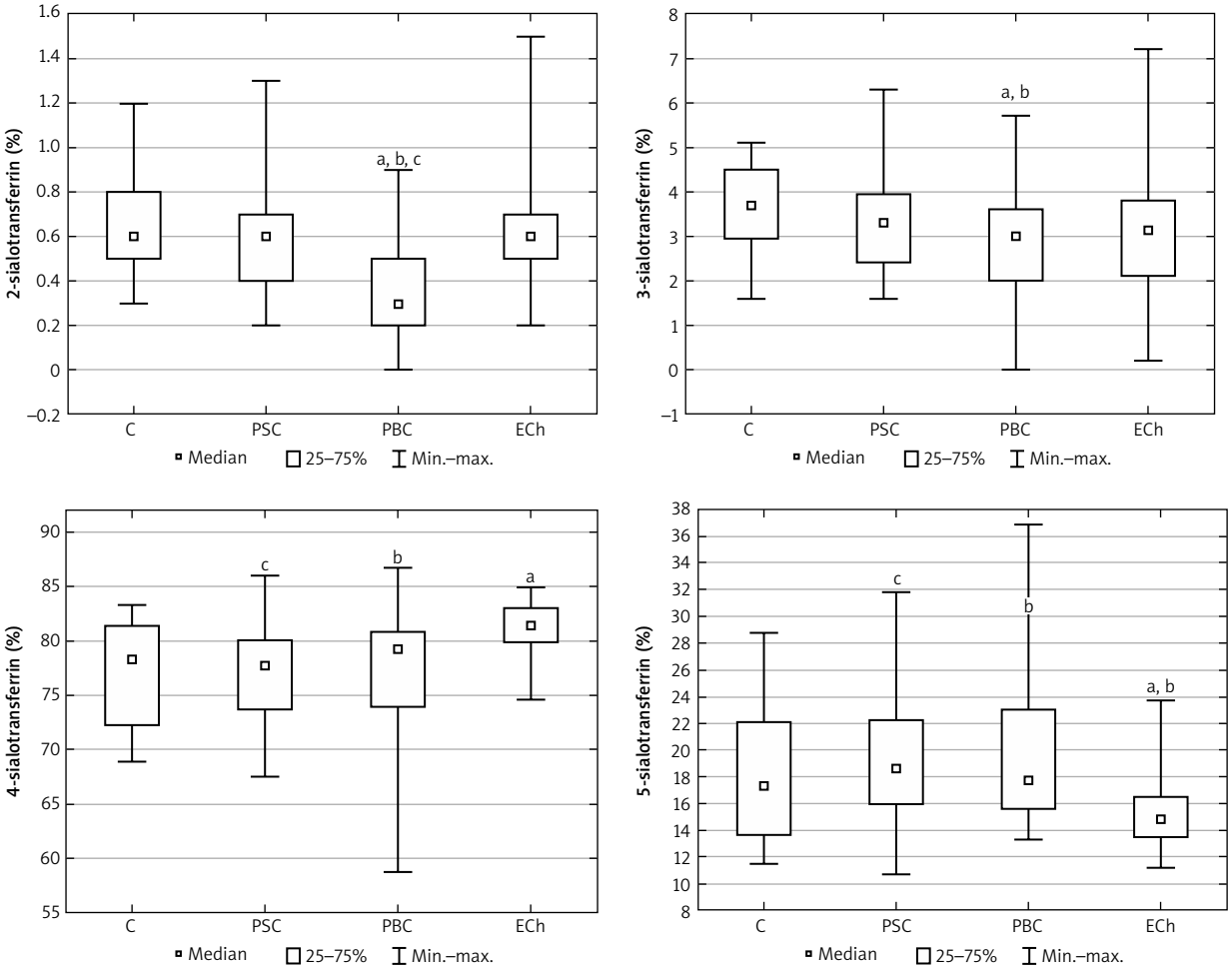

Figure 1. The concentration of disialotransferrin and trisialotransferrin in PSC and PBC. [a]Significant difference in comparison with the controls, [b]significant difference in comparison with the PSC group, [c]significant difference in comparison with the Ech group

significantly higher than that in PSC (AUC = 0.561; confidence interval: 0.406–0.716) ($p < 0.001$) and in PBC (AUC = 0.549; confidence interval: 0.399–0.700) ($p < 0.001$).

None of the transferrin isoforms changed according to staging of liver fibrosis in PSC patients ($p = 0.290$ for 2-sialotransferrin, $p = 0.924$ for trisialotransferrin, $p = 0.153$ for tetrasialotransferrin and $p = 0.092$ for pentasialotransferrin) and in PBC patients ($p = 0.184$, $p = 0.333$, $p = 0.104$, and $p = 0.040$, respectively).

Discussion

The inaccurate diagnosis of primary biliary cholangitis and primary sclerosing cholangitis may result in an inappropriate management because of different responses to ursodeoxycholic acid (UDCA) and other therapeutic methods [13–16]. Therefore, a basic and differential diagnosis are crucial for the cholangitis of different pathogenesis. Clinicians rely on the evaluation of the clinical symptoms (itching, fatigue, abdominal pain, jaundice) and laboratory results (cholestatic liver enzymes, antibodies) and, according to the results, make a decision about imaging tests [6, 7].

An organ-specific test for the liver is transferrin. Transferrin is a glycoprotein belonging to the negative acute-phase proteins synthesised in the liver [17–19]. A negative acute-phase protein is one whose concentration is reduced in liver diseases [20]. As an N-glycosylated protein, its structure shows variability depending on the attached sialic acid residues [19]. As protein glycosylation changes in the course of diseases (not only those of liver origin), the specific isoform profile in the serum could be a helpful tool in the diagnosis of different diseases [10, 11, 21, 22]. In a previous study, while testing the transferrin isoforms profile in PBC, we demonstrated changes in the concentrations of disialo- and trisialotransferrin isoforms in these patients [9]. This achievement directed our attention to other autoimmune cholestatic liver disease, i.e. PSC. To our surprise, the transferrin isoform levels did not change in PSC patients in comparison to healthy people. However, the concentration of disialotransferrin isoforms

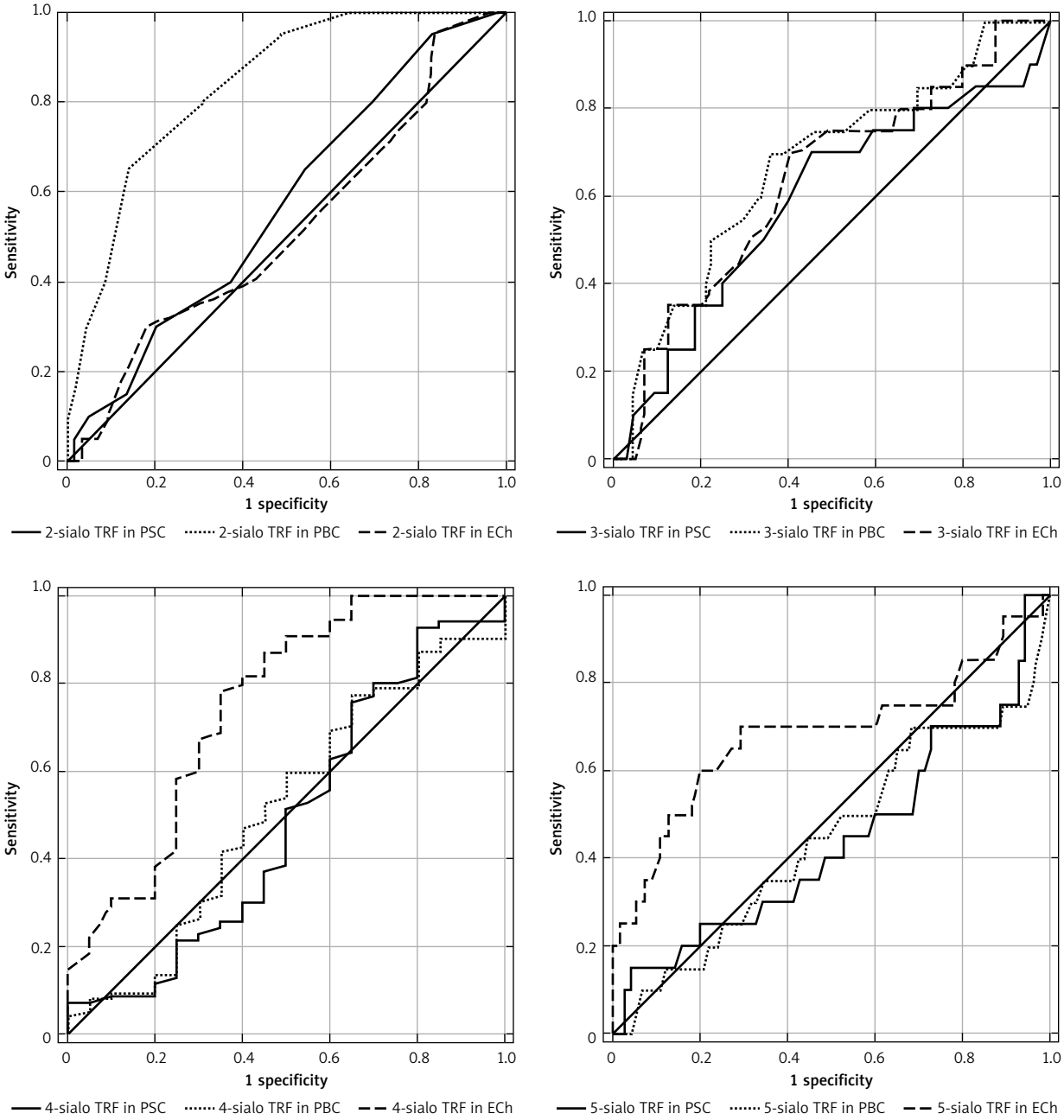

Figure 2. The area under the curves (AUCs) for transferrin isoforms in PSC, PBC asd Ech

in PSC patients was significantly higher than that in PBC. This result was approved in ROC analysis by the comparison of AUCs for PSC and PBC. This decreased disialotransferrin concentration in PBC was also lower than that in extrahepatic jaundice.

Comparing the concentration of transferrin isoforms in autoimmune liver diseases with extrahepatic cholestasis, we can see that differences also concern the higher sialylated isoforms tetra- and pentasialotransferrin. Characteristically, there is a higher concentration of pentasialotransferrin and a lower concentration of tetrasialotransferrin in PBC and PSC compared to extrahepatic cholestasis. Therefore, a completely different profile of transferrin isoforms can be seen in cholestatic autoimmune liver diseases and in extrahepatic cholestasis. This is confirmed by the analysis of ROC curves. The greatest differences in the area under the ROC curves occur for disialotransferrin between patients with PBC (reduced concentration in comparison with the controls) and PSC and Ech (lack of difference in comparison with the controls). For tetra- and pentasialotransferrin, the greatest differences occurred between patients with ECh and PSC and PBC. These results convince us that transferrin isoform determina-

tions can be used in the differential diagnosis of cholestatic liver diseases of various aetiologies.

Although the changes in transferrin isoforms in autoimmune cholestatic liver diseases are not associated with the severity of liver fibrosis, the changes in PBC seem to be associated with a higher frequency of hepatic fibrosis in PBC (84.8%) than in PSC (63.3%).

Limitations of the study included the small number of patients in the groups and the lack of subgroups of PBC without the presence of autoantibodies.

Conclusions

The profile of serum transferrin isoforms in autoimmune cholestatic liver diseases differs between them and that observed in extrahepatic cholestasis, which provides an additional opportunity to differentiate cholestatic liver diseases of different aetiologies.

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Ethical approval

Approval number: R-I-002/563/2019 for PBC study APK.002.232.2021 for PSC study.

Conflict of interest

The authors declare no conflict of interest.

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