SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

BIOMARKERS OF ABNORMAL IMMUNE RESPONSE AND DISEASE PROGRESSION IN CHRONIC LIVER DISEASES

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UNIVERSITY OF DEBRECEN

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DEBRECEN, 2021

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The Examination takes place in the Library of Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, on the 04th of June 2021 at 10 AM.

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The PhD Defense takes place online in the Library of Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, on the 04th of June 2021 at 12 AM.

If you wish to attend the presentation and the discussion, please send an email to tornai.david@med.unideb.hu before 12 PM on the 03rd of June 2021.

BACKGROUND

Cirrhosis: end stage of chronic liver diseases

Cirrhosis is the last, albeit multi-stage, dynamic phase of chronic liver diseases of various etiologies, which is extremely often identified worldwide as the cause of hospital admissions and mortality. It is the 11th most common cause of death globally and the 4th most common in Central Europe. According to the data published on the website of the Central Statistical Office, in 2019, 3303 cases of liver disease related deaths were identified in Hungary.

The disease typically affects the working age population, so in cirrhosis, the number of working years lost due to early death is much higher than in the case of cardiovascular diseases. Therefore, it is a significant burden not only for health care but also for the economy.

Alcohol abuse, non-alcoholic steatohepatitis associated with Western-type diet, and chronic viral hepatitis are the most common etiologies of liver cirrhosis. Less frequently, we identify the causal role of autoimmune processes (primary sclerosing cholangitis [PSC], primary biliary cholangitis [PBC], autoimmune hepatitis [AIH]) or metabolic diseases (Wilson's disease, hemochromatosis).

Chronic liver disease is not a stable condition but a dynamic process that progresses through stages of compensated (without complications), then decompensated cirrhosis (with complications) to end-stage liver failure when the patient's life can only be saved by liver transplantation. In cirrhosis, six subgroups can be clinically distinguished based on differences in disease progression and risk of death. In the first two (=compensated) clinical stages, patients are asymptomatic and disease-specific complications are absent. Portal blood pressure may already be elevated (in stage II, indicated by the appearance of esophageal varices), but does not reach the critical threshold (>12 mmHg). In this stage, the annual risks of both decompensation (4-12%) and death (1-3.4%) are low, and survival without liver transplantation can reach up to 15-20 years. In the advanced (III and IV; decompensated) stages, symptomatic, disease-specific complications (ascites, esophageal varices, hepatic encephalopathy [HE]) occur as the results of hepatic insufficiency due to extensive hepatocellular death and increasing portal hypertension above the clinically critical threshold. The late stage (V and VI) is the phase of recurrent acute events and cumulative complications. Annual mortality increases exponentially (20-57%) and survival without liver transplantation is expected up to 3-5 years only.

In addition, a sudden deterioration requiring hospitalization, i.e., acute decompensation (AD), may occur at any clinical stage due to an acute injury.

However, in about one third of the cases, the underlying cause cannot be determined. Factors identified so far include extensive alcohol consumption, gastrointestinal bleeding, effects of some drugs, and portal vein thrombosis. However, most often the causal role of bacterial infections is established. The most common infection is spontaneous bacterial peritonitis, which is closely associated with the presence of ascites, but pneumonia or urinary tract infection are also quite common. During AD, a significant proportion of patients may develop failure of additional organ systems, with reported short-term mortality above 50%. This clinical phenomenon, its definition, staging, and criteria for underlying organ failure are developed by the CLIF-CANONIC multicenter European prospective study. It is known in the literature as acute-on chronic liver failure (ACLF). In the CANONIC study, bacterial infection (BI) was identified in the background of about one-third of the cases, making it the most frequent trigger for ACLF. However, significant susceptibility to infection was also observed in non-BI-induced ACLF. Approximately 50% of infection-free patients (at the time of diagnosis of ACLF) developed some form of BI within 4 weeks. However, cases induced by BI or subsequently complicated by infection have been shown to be more severe, and the associated mortality is also significantly higher than in cases without infection. In the presence of BI, the risk of death in cirrhotic patients increases fourfold, regardless of the severity of the liver disease. Nearly one-third of patients die within the first month after diagnosis, while a further 30% die within 1 year.

The link between immunity disorder and bacterial infections in cirrhosis

BIs are of paramount importance both in inducing acute deterioration in patients with cirrhosis and in significantly increasing the incidence of mortality. Furthermore, advanced liver cirrhosis itself is an acquired immunodeficient condition encompassing recognition, effector and regulatory dysfunction, that results in increased susceptibility to infections and more severe episodes compared to the non-cirrhotic population.

This phenomenon is known as cirrhosis-associated immune dysfunction (CAID) syndrome, which is characterized by the dynamic and simultaneous presence of a systemic inflammatory response syndrome (SIRS) and a compensatory anti-inflammatory response syndrome (CARS). While increased inflammation dominates at the beginning of the process, parallel with the worsening of the liver disease, CARS-induced immune paralysis becomes more prominent.

Moreover, despite the increased risk of infection, the diagnosis of infections is often a pronounced challenge from both a clinical and a

laboratory point of view. In patients with cirrhosis, approximately 50% of BIs are present with an asymptomatic, nonspecific clinical picture.

Currently, CRP and PCT are the most broadly used laboratory screening markers for early detection of BIs. However, these classic parameters perform somewhat differently in comparison with the non-cirrhotic patient population for several reasons. First, the rate of bacterial translocation (BT) increases in parallel with the progression of liver disease (highest in Child-Pugh stage C). The inflammatory state it maintains may be able to significantly increase the level of markers indicative of inflammation even without manifest infection. Second, since CRP is produced primarily in the liver, the functional state of the liver influences the release of CRP, consequently, in advanced liver cirrhosis, the diagnostic accuracy of CRP decreases. Thus, the extent of CRP level elevation will not be able to reflect the severity of the infection, since the more pronounced the underlying liver failure, the less increase in CRP level can be observed at time of an overt BI. Hence, CRP shows the worst performance in case of the frailest patients. On the other hand, the concentration of PCT is affected by kidney failure or its therapy (dialysis). Acute kidney injury (AKI) is common in patients with cirrhosis, especially at time of infectious episodes. PCT has a molecular weight of 13 kDa and is eliminated predominantly by the kidneys. Therefore, in end-stage renal failure, elevated PCT concentrations can be measured even without infection due to insufficient excretion. In contrast, after renal support therapy, false-low PCT levels are detected, since molecules less than 60 kDa in size are rapidly filtered through the dialysis membrane.

New markers for the detection of cirrhosis associated bacterial infections

For these reasons, the classic clinical and laboratory parameters for the detection of BI and inflammatory reactions do not work well enough in cirrhosis. Thus, additional biomarkers are needed in this patient group for effective early detection of BI and reliable assessment of its severity and prognosis. The markers could be used to develop diagnostic laboratory panels for selecting the group of patients at highest risk for infections who may need to be closely monitored and treated with supportive and/or prophylactic antibiotic therapy. Effective planning of antibiotic prophylaxis is vital due to increasing bacterial resistance. In my own research, I investigated the serum levels of two new potential markers: ferritin and soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1).

Ferritin

Ferritin is primarily an intracellular protein, which stores up to 4,500 iron atoms per molecule in a safe but bioavailable form. It is composed of two

types of subunits, termed H and L, the ratio of which varies by organ and cell type. Ferritin secretion has been demonstrated via non-canonical pathways in macrophages, hepatocytes and Kupffer cells of the liver and it was shown to be mostly composed of the L-subunit. However, the function of circulating ferritin is still not fully understood.

Despite our tendency to consider serum ferritin as iron-poor, it usually incorporates hundreds of iron atoms, which is a substantial amount compared to the two atoms delivered by transferrin. Therefore, studies have proposed that serum ferritin might also have a role in iron transport, especially at times of increased iron demand. Ferritin is also secreted in response to inflammation and has been reported to have anti-inflammatory properties. Serum ferritin can bind to high molecular weight kininogen, preventing its cleavage and leading to the reduction in bradykinin release.

Serum ferritin level is a sensitive marker of iron homeostasis in the healthy population; reduced iron levels are associated with low ferritin, while iron overload results in higher serum ferritin concentrations, by modulating iron-response elements and their binding proteins. However, in cirrhosis, the interpretation of ferritin level is more complicated since ferritin is an acute phase protein. Therefore, its concentration also increases due to inflammation. In cirrhosis both inflammation and iron overload might be present. Accordingly, elevated hepatic and serum ferritin levels are consistently reported in chronic liver diseases.

Iron overload in cirrhosis seems to be due, at least in part, to reduced hepcidin levels caused by decreased synthetic capacity of hepatocytes. The role of hepcidin is to inhibit iron uptake by ferroportin in the basal membrane of enterocytes. Therefore, decreased hepcidin concentration leads to increased iron uptake. Additionally, infection/inflammation also decreases hepcidin levels. Endotoxemia, driven by BT, is common in cirrhosis and maintains a chronic inflammatory state in these patients. At the same time, since cirrhosis is an immune suppressed condition, especially in the later decompensated state of the disease, infections appear more frequently and show a more severe course in cirrhotic patients than in the healthy population. Thus, in cirrhosis, hepcidin synthesis can be suppressed both chronically (deteriorating liver function and chronic inflammation) and acutely (infections), leading to increased iron absorption and elevated free iron levels. Iron, loaded in the liver cells or macrophages, was shown to be toxic, potentiates oxidative stress and induces peroxidation, resulting in further liver cell damage.

On the other hand, acute and chronic bleedings are common in cirrhotic patients. Portal hypertension and instable hemostasis increase the risk of variceal bleeding, portal hypertensive gastropathy, gastric antral vascular ectasia and peptic ulcer bleeding. Malabsorption may also be present in

cirrhotic individuals contributing to reduced iron storages. Iron deficiency in return was shown to contribute to lipid, glucose and nutrient metabolic dysfunction, as well as induction of cell apoptosis.

All of these complex processes are reflected in serum ferritin levels. The role of ferritin has already been studied in AD, where an association was found between elevated concentrations and higher short-term mortality. However, the association of serum ferritin levels with long-term outcomes has not been studied in stable outpatients with cirrhosis.

Soluble Triggering receptor expressed on myeloid cells-1 (sTREM-1)

TREM-1 is a surface receptor that amplifies inflammation induced by toll-like receptors (TLRs) by further increasing the production of proinflammatory cytokines, and is expressed on neutrophils, monocytes and macrophages. TREM-1 signaling leads to phosphorylation and activation of Spleen Tyrosine Kinase, which has been indicated as a major regulator in inflammatory processes of different liver diseases, while interfering with this pathway ameliorated these conditions. However, role of TREM-1 has also been indicated in various other inflammatory diseases, such as sepsis, atherosclerosis, colitis and hepatocellular carcinoma (HCC).

While the ligand of TREM-1 is still unknown, it was observed that BI and challenge with lipopolysaccharide or lipoteichoic acid increase TREM-1 expression. Simultaneously with its cell surface induction, a soluble form of the molecule (sTREM-1) accumulates in the circulation during inflammation. The production of sTREM-1, whether it is a splice variant or the result of proteolytic cleavage, is still a matter of debate but both mechanisms are likely to occur. sTREM-1 acts as a decoy receptor by competing for the unknown TREM-1 ligand, hence, mitigate the activation of membrane bound TREM-1. sTREM-1 concentration has been proved to be a valuable marker in pneumonia and sepsis. Since circulating sTREM-1 mainly originates from myeloid cells, liver failure and its severity might not alter the diagnostic application of sTREM-1 in cirrhosis, which could be an important advantage in clinical practice.

Primer sclerosing cholangitis (PSC)

PSC is a chronic cholestatic liver disease characterized by persistent, progressive biliary inflammation and fibrosis, eventually leading to cirrhosis in a significant proportion of the patients. While the etiological background of the disease is poorly understood, intestinal-liver interactions appear to play an important role in its development. It seems that the interplay between certain genetic factors (such as Human Leukocyte Antigens) and the gut-microbiome

leads to the development of dysregulated immunological pathways by an autoimmune reaction to hitherto unknown antigen(s). This may explain the frequent association of PSC with inflammatory bowel disease (IBD). Most findings of genetic research support a disruption of T-cell function. Intestinal antigens significantly induce antigen presentation of MHC complexes located on the surface of antigen-presenting cells to T-cells. After clonal expansion, T-cells can reach both the intestinal tract and the liver. Abnormal T-cell activation results in increased release of various cytokines (such as transforming growth factor-β) that are involved in the development of fibrosis. Activation of B cells is indicated by the frequent appearance of BT-associated serological antibodies (e.g., anti-neutrophil cytoplasmic antibody [ANCA]).

Endotoxin exposure and inflammation disrupt the tight junctions of cholangiocytes, making these cells even more vulnerable to further damaging factors (e.g., bile acids). Cross-communication between damaged cholangiocytes, hepatic stellate cells and portal myofibroblasts with hitherto unclear molecular mechanisms results in the development of concentric fibrosis around the bile ducts in PSC, leading to a significant increase in the risk of bile duct stenosis and cholangiocarcinoma (CCA).

The disease is difficult to diagnose because we do not have PSC specific biomarkers. The diagnosis of PSC is established based on the cholangiographic image that shows multiple stenoses and dilatations along the bile ducts, usually in patients whose laboratory tests indicate persistent cholestasis.

Therapeutic options are also limited, curative medication is not available. We cannot even prevent the progression of the disease and the development of complications and end-stage liver failure. Once cirrhosis is developed, liver transplantation is the only option, however, in a quarter of cases, PSC recurs in the graft. Dilation and stenting of the dominant stenoses in the biliary tract offer an opportunity to treat recurrent bacterial cholangitis, but this procedure is accompanied by additional liver damage. The life expectancy of patients with PSC is significantly shorter than in the average population, and mortality is approximately four times higher.

IgA type autoantibodies against pancreatic glycoprotein 2 (GP2)

GP2 is secreted to the pancreatic fluid in significant amounts from zymogenic granules of the exocrine pancreas and functions as a soluble receptor for type 1 bacterial pili with fimbrial adhesin H (FimH) component. However, the protein is also expressed in a membrane-bound form on the

apical surface of M-cells in Peyer's patches, where GP2 plays a role in the detection of bacterial antigens. GP2 is thus essential in the development of the mucosal immune response against intestinal bacteria with FimH-component. Its role is likely to maintain a balance between tolerance to commensal bacteria and the immune response to pathogenic strains.

Enhanced BT may be associated with increased antimicrobial antibody production, which may lead to the development of autoantibodies if the process becomes pathological. Autoantibody formation against acinar cells of the exocrine pancreas have long been described in Crohn's disease (CD) and considered one of its various serological hallmarks. Recently, one of the target antigens of pancreatic autoantibodies have been identified as GP2. Previously, we demonstrated in a large cohort of patients with IBD, that the presence of anti-GP2 antibodies was associated with concomitant PSC and also with faster disease progression in patients with CD. Interestingly, only IgA but not IgG antibodies were related to the development of complications.

Antibodies of the IgA isotype play a particularly important role in the immune defense of the intestinal mucosa. Although assessment of serum IgG isotype autoantibody levels is used generally in laboratory testing of systemic autoimmune diseases, in case of intestinal mucosal involvement, IgA-type antimicrobial and autoantibodies may be more informative in the evaluation of the disease state and pathology.

In humans, the vast majority of total serum IgA belongs to the IgA1 subtype, while IgA2 is only a fraction of the whole. Based on previous studies, an increase in the ratio of IgA2 and the concomitant presence of the secretory component (SC) are considered as evidence of mucosal IgA secretion. In healthy individuals, the percentage of SC containing IgA in terms of the total IgA pool is less than 1%, which may be explained by its mechanism of formation.

The epithelial cells of the intestinal mucosa take up the dimeric IgA molecule by the poly-IgA receptor, and after intracellular transport, secrete it to the mucosal surface. In the process, the receptor is cleaved, and a piece (SC) remains covalently bound to the antibody. Together they form the so-called secretory IgA (sIgA). The complex secreted into the lumen has a dual function. On the one hand, it neutralizes certain microbial antigens, preventing their interaction with the intestinal epithelium. This process is called immune exclusion. On the other hand, sIgA is also involved in the reverse transport of antigens (from the intestinal lumen to the immune tissue), a process of paramount importance for proper immune functions. After retrieval, sIgA

antibodies also enter the thoracic duct and reach the systemic circulation. Thus, they can be detected in the serum.

Since the association between PSC development and intestinal mucosal damage (as well as the concomitant BT) is supported by a number of experimental data, IgA-type autoantibody assays are expected to be relevant in this disease, too. Therefore, in our work we determined the IgG and IgA isotype of the anti-GP2 antibodies and evaluated their clinical significance in PSC.

AIMS

We tested 3 different biomarkers in chronic liver patients. We examined the suitability of the molecules for assessing disease severity and prognosis and for detecting associated bacterial infections or predicting their development.

1. In a cohort consisting exclusively of cirrhotic outpatients, we tested the association between serum ferritin levels and disease severity. We investigated ferritin's predictive ability for disease progression in compensated patients, BI development in decompensated patients, and mortality in the entire population during a 2-year-long follow-up.
2. We examined the association between serum levels of sTREM-1 and disease severity, presence of BI, and 90-day mortality of patients with BI in a cirrhosis cohort including both outpatient and hospitalized patients with AD.
3. We examined the presence of different anti-GP2 autoantibody isotypes in a cohort of PSC patients. Furthermore, we determined the association of the marker with the severity of the disease and assessed its suitability in predicting transplantation-free survival time during a 10-year-long follow-up.

PATIENTS AND METHODS

Study populations

Cirrhosis

We recruited adult patients with established diagnoses of cirrhosis of different etiologies from the outpatient clinic at our gastroenterology tertiary care referral center during regular or unscheduled follow-up visits and from the inpatient ward at the events of hospitalization with AD episodes.

Between May 2006 and October 2011, 244 well-characterized cirrhotic outpatients with 2 years of clinical follow-up were included to the ferritin study.

We recruited 269 cirrhotic patients for the sTREM-1 study between December 2014 and June 2016. Serum samples of 172 outpatients and 97 hospitalized AD subjects were collected. 38 patients provided blood at the time of both outpatient visits and at the events of acute decompensation for paired comparison. These outpatient samples were excluded from other analyses.

The diagnosis of cirrhosis was based on the combination of clinical, biochemical, imaging, and when available, histological data.

Blood samples, routine laboratory data and detailed clinical phenotype were captured at inclusion. Clinical data were determined by an in-depth review of the patients' medical records using a structured interview. Medical records were retrospectively analyzed for the period prior to the observational follow-up study. At enrolment, disease severity (assessed by liver-oriented scores: Child-Pugh and MELD) and the clinical stage of the diseases were determined. Clinical scores, presence and type of AD, BI and ACLF were assessed according established diagnostic criteria and clinical events were recorded during follow-up. AD was defined by acute development of large ascites (grade II/III), acute hepatic encephalopathy (HE), acute variceal bleeding and/or presence of BI warranted hospital admission. BI development was based on compatible clinical symptoms, imaging findings and laboratory data.

Collected data were transferred and stored in our database. At the end of the study period, all clinical data were extracted for further analysis.

Primer sclerosing cholangitis

We performed an observational cohort study among adult and pediatric PSC patients recruited in Hungarian referral hepatology centers. In total, 65 well-characterized PSC patients (55 adults and 10 children) with a complete clinical follow-up were included between January 2006 and December 2007.

Diagnosis of PSC was based on clinical, biochemical, serological and cholangiographic (magnetic resonance or endoscopic imaging) features or, when indicated, on histological findings. Patients with secondary sclerosing cholangitis or any concomitant malignant disease were excluded. Blood samples and detailed description of clinical phenotypes were obtained at inclusion. Medical records were retrospectively analyzed for the period prior to the prospective follow-up. At enrolment, revised Mayo risk score was calculated and biochemical analyses were performed using standard routine laboratory tests.

PSC patients were enrolled into a prospective follow-up study, where treating physicians registered laboratory data, imaging and endoscopic

findings, medical treatment, date and type of complications (cirrhosis, colorectal cancer, biliary tract cancer: cholangiocarcinoma (CCA), gallbladder cancer or cholangitis) during regular outpatient follow-up visits and inpatient stays. Adverse outcome was defined as need for orthotropic liver transplantation (OLTx) and/or liver-related death (composite end-point). Follow-up for a particular patient was terminated if adverse outcome occurred or there was no further record available. Collected data were transferred and stored in a database from where it was retrieved after the study period for analysis.

Control groups for PSC

Healthy controls and two disease control groups (patients with inflammatory bowel diseases [IBD] and chronic liver diseases [CLD]) were included.

The healthy control group consisted of 100 age-matched individuals (male/female: 45/55, age: 30 years [21–40]) selected from consecutive blood donors in Debrecen. Control subjects did not have any known gastrointestinal or liver diseases.

The inflammatory bowel disease control group consisted of a previously reported patient cohort (CD: 257, male/female: 109/148, age: 25 [19–33] years and ulcerative colitis [UC]: 170, male/female: 78/92, age: 34 [23–44] years).

The chronic liver disease control group consisted of patients with primary biliary cholangitis without cirrhosis (PBC, n=102, male/female: 4/98, age: 59 [52–66] years), chronic hepatitis C virus (chrHCV, n=119, male/female: 50/69, age: 55 [47–65] years) and alcoholic liver cirrhosis (aLC, n=267, male/female: 147/120, age: 58 [51–66] years). The diagnosis of PBC was based on biochemical evidence of cholestasis, serum anti-mitochondrial antibodies (AMA) and/or PBC-specific AMA-M2 positivity, compatible histology, and the exclusion of extrahepatic cholestasis. The diagnosis of chronic HCV was based on positive HCV ribonucleic acid tests, elevated liver function assays (2xULN for more than 6 months) and compatible liver biopsy, if available. The diagnosis of cirrhosis was based on clinical, biochemical, imaging, and, when available, histological data.

Serological analysis

Blood samples were obtained at enrolment from each patient. Routine laboratory parameters (liver enzymes, liver and kidney function tests, CRP, blood cell counts) were determined at the Department of Laboratory Medicine, Clinical Center, University of Debrecen.

Ferritin level was measured by a two-site chemiluminescence immunoassay (Roche, Basel, Switzerland) using the Cobas e602 analyzer (Roche) in our routine laboratory unit. The assay sensitivity limit was 0.5 μg/L. As there is no consensus on pathologic ferritin levels in cirrhotic patients, in our study low (<40 μg/L) and high (>310 μg/L) ferritin levels were determined arbitrarily corresponding to the patients' 25^{th} and 75^{th} serum level percentile (i.e., 1^{st} and 4^{th} quartile [Q1 and Q4]).

To evaluate the association between serum ferritin levels and other parameters of hematopoiesis, we categorized patients into anemic and non-anemic groups according to their hemoglobin and hematocrit values. In our routine diagnostic laboratory, the reference ranges of hemoglobin and hematocrit are 115-150 g/L and 0.35-0.47 for females and 130-165 g/L and 0.39-0.50 for males, respectively. Patients were categorized into the anemic group if at least one of these parameters was below the lower limit of the reference range.

Experimental measurements were performed from sera kept frozen at -70°C after isolation. Serum level of sTREM-1 was determined by solid-phase enzyme-linked immunoassay, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Samples were measured in duplicates on the same plate, and the mean values were used. Between runs coefficients of variation was 9%. The limit of detection was 7 pg/mL.

IgA and IgG type anti-GP2 antibodies were detected in sera using commercially available cell-based IIFT according to the test instructions (CIBD Mosaic, EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). A specific fluorescence at a dilution of 1:10 or higher was considered positive as recommended by the manufacturer.

Secretory subtype of total IgA (sIgA) was detected by an in-house sandwich ELISA in PSC and healthy controls.

We used a GP2-coated bead-based in-house flow cytometric immunoassay for IgA subtype analysis and to determine the presence of secretory component (SC) on anti-GP2 IgA antibodies in sera. The assay was performed in anti-GP2 IgA-positive sera of PSC (n = 19) and Crohn's disease patients without PSC (n = 12) detected by IIFT, and verified by ELISA test (anti-GP2 IgA, GA Generic Assays, Dahlewitz/Berlin, Germany). Anti-GP2 IgA-negative sera of healthy subjects (n = 20) served as controls.

All serological assays were performed at the Department of Laboratory Medicine in a blinded fashion without prior knowledge of the patient's clinical information.

Statistical analysis

Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as median and interquartile range (IQR [lowest 25%-highest 25%]) and compared with Mann-Whitney U test for two groups or Kruskal-Wallis H test with Dunn's multiple comparison post hoc analysis for three or more groups. Spearman's nonparametric rank correlation test was used to determine correlations. Ability of different variables to discriminate between patients with or without infection as well as survivors and non-survivors in patients with BI were assessed by receiver operating characteristics (ROC) curve analysis plotting sensitivity% vs. 100-specificity%. Area under the curve (AUROC) and corresponding 95% confidence intervals (CI) were calculated. Youden index (maximum value of sensitivity + specificity) was calculated to estimate the best discriminative threshold. Sensitivities, specificities, positive predictive values (+PV) and negative predictive values (-PV) were calculated at the best discriminative threshold of the variables. ROC curves were compared with the method of DeLong et al. ROC of the composite score was validated by bootstrapping the CI using 10,000 iterations. Kaplan–Meier analysis was used to calculate the cumulative probability of mortality. Differences in observed probabilities were assessed with the log-rank test. The association between categorical clinical variables or serum marker levels and mortality during follow-up was assessed with univariate Cox-regression analysis. For adverse outcomes with competing events, we used the Aalen-Johansen estimator, a modified version of the Kaplan-Meier analysis, that takes competing outcomes into account, to plot the cumulative incidence function. The association between these events, clinical factors and serum ferritin levels was calculated by univariate Fine-Gray proportional hazard regression. Multivariate analyses were performed with backward elimination procedure and likelihood ratio test to identify independent predictors. Associations are provided as hazard ratio (HR) or sub-distribution hazard ratio (sHR) with 95% confidence intervals (CI). For the Fine-Gray test, we utilized R 3.5.1 supplemented with the EZR package, R commander and RcmdrPlugin.EZR plugin. For all other statistical analyses and graphical presentation, the SPSS 25.0 (SPSS, Chicago, IL) and GraphPad Prism 8.4 (San Diego, CA) programs were used. A 2-sided probability value of <0.05 was considered to be statistically significant.

RESULTS

Ferritin

Association of ferritin with clinical and laboratory parameters

Serum levels of ferritin ranged from 1.5 to 1954.6 μg/L in our cirrhotic patient cohort. Increased ferritin levels were found in males compared to females (median [IQR]: 152.6 [60.9-420.4] vs. 75.0 [30.3-223.5] μg/L, $p<0.001$), and in ≥50 years old patients compared to younger ones (median [IQR]: 142.9 [47.0-333.0] vs. 67.9 [24.8-170.8] μg/L, $p=0.002$). When we categorized our patients according to disease severity as described by Child-Pugh stages (median [IQR]: A: 92.0 [34.2-303.4]; B: 131.1 [44.2-289.0]; C: 340.1 [227.1-548.4] μg/L) we only observed statistically significant difference between the A and C groups ($p=0.041$). Furthermore, we didn't detect significant differences in serum ferritin levels between patients with or without ascites (median [IQR]: 153.4 [45.4-370.7] vs. 95.0 [36.3-297.0] μg/L, $p=0.139$), in the absence or presence of HCC (median [IQR]: 103.8 [39.3-301.4] vs. 149.1 [42.7-399.6] μg/L, $p=0.246$), and with alcoholic or non-alcoholic etiology (median [IQR]: 133.2 [42.7-363.1] vs. 92.0 [35.0-271.9] μg/L, $p=0.208$).

Importantly, ferritin levels were significantly lower in patients who had a prior variceal bleeding episode compared to those who did not have such an event (median [IQR]: 43.1 [19.8-125.6] vs. 146.6 [52.7-358.3] μg/L, $p<0.001$). However, when we divided our patients according to the presence or absence of anemia as described in the Methods section, we did not find significant differences between the two groups (median [IQR]: 92.5 [31.2-301.4] vs. 132.4 [43.8-320.0] μg/L, respectively; $p=0.144$).

Next, we investigated the association between serum ferritin level and other laboratory parameters (i.e., continuous variables). While ferritin levels correlated with markers of hematopoiesis and iron homeostasis in both compensated and decompensated patients, the indicators of inflammation (CRP and white blood cell counts) showed correlation with ferritin levels only in patients with previous decompensation. Similarly, disease severity measures (Child-Pugh score and MELD) correlated with ferritin levels only in the decompensated group.

Low serum ferritin level is associated with increased 2-year incidence of developing a decompensated clinical stage

Of 244 patients, 143 (58.6%) experienced at least one form of decompensation episodes (ascites development, variceal bleeding, HE) prior to enrolment (decompensated patients) and 101 (41.4%) had medical histories free from these events (compensated patients).

In patients with compensated cirrhosis, we investigated the impact of serum ferritin levels on the development of decompensated clinical stage during the two-year follow-up period. We registered such events in 20 of our previously compensated patients (19.8%), of which 4 were associated with BI. As the first AD, 15 patients had ascites, 8 HE and 6 variceal bleeding. In 3 cases, all three events developed simultaneously, in 2 of them, ascites occurred in combination with HE, while in one case, ascites and variceal bleeding appeared together.

We summarized these episodes and plotted Aalan-Johansen graphs. Low (Q1) ferritin levels were associated with an increased incidence of first appearing AD events (39.4%) compared to higher values (Q2-4; 16.2% p=0.023) during the two-year follow-up.

In univariate Fine-Gray proportional hazard regression analysis, low (Q1) serum ferritin concentration was found to be significantly associated with the development of first AD events (sHR: 2.782, CI: 1.156-6.696, p=0.022) besides 5-point increase in MELD score, presence of comorbidities, HCC, higher than 10 mg/L CRP serum level and anemia. In the subsequent multivariate Fine-Gray proportional hazard regression model with backward elimination, 5-point increase in MELD score, presence of HCC, anemia and low (Q1) serum ferritin concentration (sHR: 3.762, CI: 1.616-8.760, p=0.002) were independent risk factors of first AD development.

High serum ferritin level is associated with increased 2-year development of BI

In decompensated cirrhotic patients, we evaluated the incidence of BI development, as it is the most frequent cause of morbidity and mortality in this considerably immunocompromised state of the disease. Seventy of these patients developed at least one AD episode during follow-up, and 45 of these cases were attributed to BIs. Therefore, in this population, we took AD events both with and without BI as well as mortality into account when plotting Aalan-Johansen graphs. Corresponding to an increased inflammatory state, in this population, high (Q4) ferritin levels were associated with an increased incidence of BI development (50.2%) compared to lower values (Q1-3; 30.9%, p=0.035) during the two-year follow-up.

In univariate Fine-Gray proportional hazard regression analysis, age of 50 or more, female sex of the patients, presence of ascites, previous BI episode, >10 mg/L CRP serum level and high (Q4) serum ferritin concentration (sHR: 1.991, CI: 1.054-3.758, p=0.034) were associated with the development of BI. Multivariate Fine-Gray regression model indicated that female sex of the patients, presence of ascites, previous BI episode and high

(Q4) serum ferritin concentration (sHR: 2.335, CI: 1.193-4.568, p=0.013) were independent risk factors of 2-year of BI development.

High serum ferritin level is associated with increased 2-year mortality

We evaluated the potential impact of serum ferritin level on mortality in the whole cohort as well as in both compensated and decompensated patient populations. High ferritin levels were found to be associated with increased mortality rate in all these groups assessed with Kaplan-Meier analysis.

We found the same association even when we divided compensated patients according to who did and did not develop decompensated disease stage during follow-up. In both subpopulations, mortality showed higher incidence in patients with high (Q4) ferritin levels. However, due to the low number of events in the group that remained compensated, this association reached statistical significance only in patients who developed decompensated clinical stage.

Univariate Cox regression analyses also indicated association between high (Q4) ferritin levels and 2-year mortality in every group (all: HR: 2.209, CI: 1.269-3.844, p=0.005; compensated: HR: 2.903, CI: 1.052-8.012, p=0.040; decompensated: HR: 2.251, CI: 1.146-4.424, p=0.019). Relevant clinical and laboratory factors were also evaluated.

Multivariate Cox regression model revealed that high (Q4) ferritin level was an independent risk factor of 2-year mortality in the whole patient cohort (HR: 2.143, CI: 1.174-3.910, p=0.013) besides disease severity indicators and presence of HCC. However, when we split the cohort into compensated and decompensated patients, we observed this association only in the compensated cirrhosis group (HR: 4.367, CI: 1.466-13.009, p=0.008). In decompensated patients, clinical factors were found to be stronger predictors of mortality.

<u>Soluble Triggering Receptor Expressed on Myeloid cells-1</u>

sTREM-1 levels are associated with disease severity and presence of infection

Serum values of sTREM-1 ranged from 8 to 3016 pg/mL in the total patient population and it proportionally increased with Child-Pugh stages (A: median [IQR]: 203 [124-332], B: 350 [211-555], C: 725 [452-986] pg/mL; p<0.0001 for all). Similarly, we measured significantly higher sTREM-1 levels in AD patients than in stable outpatients (median [IQR]: 509 [323-850] vs. 211 [130-366] pg/mL; p<0.0001). This elevation was detectable compared to outpatients with both compensated (median [IQR]: 190 [123-325] pg/mL, p<0.0001) and decompensated (median [IQR]: 235 [148-397] pg/mL, p<0.0001) clinical stages, while there was no difference between the two

groups of outpatients. We made the same observation when we categorized our outpatients according to disease severity: Child-Pugh A (median [IQR]: 202 [123-330] pg/mL, p<0.0001) Child-Pugh B&C (median [IQR]: 265 [167-455] pg/mL, p=0.0004). Among AD patients Child-Pugh C stage patients had significantly elevated sTREM-1 levels (median [IQR]: 787 [475–1,013] pg/mL) compared to both Child-Pugh A and B stage patients (median [IQR]: A: 286 [128-830] pg/mL, p=0.0106; B: 401 [279-659] pg/mL, p=0.0002). Furthermore, sTREM-1 concentration was significantly increased in AD patients with BI (median [IQR]: 711 [442-1026] pg/mL) compared to AD patients without infection (median [IQR]: 323 [205-507] pg/mL, p<0.0001). Consistently, among patients with BI, presence of sepsis was associated with increased sTREM-1 levels (median [IQR]: 636 [395-989] vs. 946 [774-1,192] pg/mL, p=0.0072). However, in the absence of BI, AD cases showed increased sTREM-1 levels only compared to compensated clinical stage or Child-Pugh A severity outpatient groups. We measured higher sTREM-1 levels in samples of patients with BI who also had acute kidney injury (AKI) at the time of enrolment (median [IQR]: 1,062 [764-1,583] pg/mL) compared to patients without AKI (median [IQR]: 612 [376-827] pg/mL, p=0.0214) or without both BI and AKI (median [IQR]: 323 [181-508] pg/mL, p<0.0001). Finally, patients who developed ACLF had higher sTREM-1 levels compared to AD patients without ACLF (median [IQR]: 850 [589-1149] vs. 401 [288-669] pg/mL, p<0.0001).

Thirty-eight patients had both outpatient and AD samples (20 without and 18 with infection). In paired analysis, patients with BIs had statistically significant increase in sTREM-1 level compared to their own samples at the time of an outpatient visit (median [IQR]: 294 [235-381] vs. 452 [338-849] pg/mL, p<0.0001) while we did not observe such elevation in case of AD samples without BI.

Significant correlation was found between sTREM-1 level and laboratory markers of inflammation, impaired renal and liver function as well as liver-oriented scores (Child-Pugh, MELD and CLIF-AD). Notably, all correlations were found to be stronger in the AD population compared to outpatients.

A new infection score based on sTREM-1, CRP and the presence of moderate/severe ascites has significantly better performance in identifying BI among AD patients than CRP alone

Next, we investigated the accuracy of sTREM-1 and CRP to identify BI in patients with cirrhosis. First, as a reference, we compared AD patients with BI to stable outpatients using ROC curve analysis. Discriminative performance of both sTREM-1 (AUROC: 0.888, CI: 0.844-0.931, p<0.0001,

overall accuracy: 81.2%, sensitivity: 80.4%, specificity: 83.7%, +PV & -PV: 61.6% and 92.9% respectively at a best discriminative cut-off of >429 pg/mL) and CRP (AUROC: 0.904, CI: 0.859-0.949, p<0.0001, overall accuracy: 85.6%, sensitivity: 92.9%, specificity: 81.6%, +PV & -PV: 63.4% and 97.1% respectively at a best discriminative cut-off of >9.1 mg/L) was excellent with a not significantly better performance of CRP. However, when we used these markers to separate infective patients from AD patients without BI both markers lost a great value of their discriminative power, but since CRP lost more (ΔAUROC: CRP: -0.113; sTREM-1: -0.084), this time sTREM-1 provided a not significantly better performance (sTREM-1: AUROC: 0.804, CI: 0.711-0.897, p<0.0001, overall accuracy: 73.2%, sensitivity: 60.7%, specificity: 90.2%, +PV & -PV: 89.5% and 62.7% respectively at a best discriminative cut-off of >642 pg/mL; CRP: AUROC: 0.791, CI: 0.702-0.881, p<0.0001, overall accuracy: 72.2%, sensitivity: 55.4%, specificity: 95.1%, +PV & -PV: 93.9% and 60.9% respectively at a best discriminative cut-off of >36.2 mg/L).

Hence, we aimed to combine these two markers to improve diagnostic performance using logistic regression. We got the best results by combining the natural logarithm of sTREM-1 with the best discriminative cut-off of CRP. After the addition of a clinical factor, the presence of moderate/severe ascites (which is detectable by physical examination), our new AD infection (ADI) score (=1.016 x $\ln$(sTREM-1)+3.138$_{[\text{if CRP>36.2 mg/L}]}$+1.994$_{[\text{if ascites is present}]}$) had significantly higher AUROC than the routinely used CRP alone (AUROC: 0.878, CI: 0.812-0.944, p<0.0001, overall accuracy: 79.4%, sensitivity: 76.8%, specificity: 82.9%, +PV & -PV: 86.0% and 72.3% respectively at a best discriminative cut-off of >8.38; ΔAUROC: 0.087, CI: 0.004-0.168, p=0.0394). Finally, we used bootstrapping as an internal validation for the CI of the ADI-ROC curve, which confirmed the performance of the new score (bootstrapped CI: 0.798-0.932).

Another, simpler approach for combination of CRP and sTREM-1 is to define the severe cases as "at least one positive test of the two" (by the above defined cut-off values). Using this way of evaluation ROC analysis cannot be performed but the overall accuracy: 77.3%, sensitivity: 71.4%, specificity: 85.4%, +PV & -PV: 87.0% and 68.6% respectively, are comparable to the new ADI score**.**

High sTREM-1 level is an independent predictor of 90-day mortality in patients with BI

Of the fifty-six patients with BI, twenty-eight (50%) died during the 90-day follow-up. sTREM-1 levels at admission were significantly higher in non-survivors than survivors (median [IQR]: 497 [376-938] vs. 850 [695-

1,261] pg/mL, p=0.0023). Prognostic accuracy of sTREM-1 level for predicting 90-day mortality in patients with BI was established by ROC analysis (AUROC: 0.733, CI: 0.598-0.869). The best discriminate threshold of sTREM-1 level, estimated by the Youden-index, was >660 pg/mL with sensitivity of 82.1%, specificity of 64.3%, +PV and -PV of 69.7% and 78.3%, respectively. Using this derived cut-off in Kaplan-Meier analysis of patients with BI, patients with high sTREM-1 concentration (>660 pg/mL) had significantly higher risk of 90-day mortality when compared to those with low sTREM-1 levels (Log-Rank p<0.0001).

Clinical scores are well established and commonly used in continuous fashion for predicting mortality. On the other hand, from a clinician's point of view single cut-off values - defining positive/negative "binary" outcomes - might be more useful regarding individual markers due to the simplicity of this approach. To avoid the statistical bias borne by comparing cut-offs to continuous scores we established predictive values of MELD and CLIF-AD scores, CRP and sTREM-1 as both continuous and categorical variables (based on their best discriminative cut-off values).

In the univariate Cox-regression analysis, both the natural logarithm (ln) of sTREM-1 (HR: 2.606, [95%CI: 1.468-4.627], p=0.001) and high sTREM-1 levels (>660 pg/mL) (HR: 5.095, [95%CI: 1.920-13.524], p=0.001) showed association with 90-day mortality besides the following clinical factors: MELD and CLIF-AD scores as well as high values (>18 and >60, respectively), ACLF grade, presence of pneumonia as site of infection, appearance of secunder infection, gastrointestinal bleeding, ln(CRP) and high CRP levels (>36.5 mg/L). In our first multivariate Cox regression model, including the above mentioned factors as continuous variables, only ACLF grade remained independent predictor of 90-day mortality, however ln(sTREM-1) almost reached statistical significance (HR: 2.014, [95%CI: 0.982-4.132], p=0.056) as the second strongest predictor. The second multivariate Cox regression model including categorical variables indicated that high sTREM-1 level (HR: 2.941, [95%CI: 1.009-8.573], p=0.048) and ACLF grade together remained independent predictors of 90-day mortality during BI episodes.

Anti-pancreatic glycoprotein 2 autoantibodies

The occurrence of GP2-specific autoantibodies is significantly more common in PSC than in control groups

Cases positive for anti-GP2 antibody were almost exclusively of IgA isotype, with IgG positivity found only in 7% of CD controls. Overall, 30.8% (20/65) of PSC patients were positive for the presence of GP2-specific IgA autoantibody, which was significantly more common compared to the control

groups (healthy: 0.0%, CD: 6.2, UC: 0.0%, aLC: 4.9%, chrHCV: 4.2%, PBC: 3.9%; p<0.001 in each case).

The presence of anti-GP2 IgA is associated with a more severe disease phenotype in PSC

Anti-GP2 antibodies demonstrated no association with gender and younger age at diagnosis. Several laboratory and clinical parameters, indicating more severe disease were significantly higher in the presence of anti-GP2 IgA antibody. Patients with anti-GP2 IgA antibodies had a significantly shorter disease duration (4 [2–7] vs. 7 [3–10] years, p=0.009), and increased Mayo risk score. All liver enzymes were significantly elevated, while albumin level was decreased in anti-GP2 IgA positive cases compared to anti-GP2 negative ones. There was an elevated occurrence of cirrhosis in anti-GP2 IgA-positive patients (35% vs. 11.1%, p=0.022).

The presence of anti-GP2 IgA is associated with a risk of disease progression in PSC

Seven patients underwent orthotopic liver transplantation (OLTx) during the follow-up period. In all cases the indication was the development of end-stage liver disease. Six patients died due to liver-related complications, three out of six deaths occurred after OLTx, therefore the composite end-point (OLTx and/or liver-related death) occurred in a total of 10 patients. One patient died due to acute myocardial infarction, this case was censored at time of event. Median follow-up from inclusion was 2,632 [IQR: 286–3,022] days. Development of colon cancer occurred in 2, while biliary tract cancer in 1 patient. Nine patients had at least one episode of cholangitis.

We analyzed the association of clinical variables and anti-GP2 IgA with poor disease outcome. In Kaplan-Meier analysis, the median time to OLTx and/or liver-related death was 490 days (IQR: 49–1,033). Mayo risk score and the presence of cirrhosis (Log-rank p<0.001 and =0.007, respectively), but not gender (Log-rank p=0.441), age at onset (Log-rank p=0.884), disease location (Log-rank p=0.722) or concomitant IBD Log-rank p=0.432) were significantly associated with faster disease progression.

Positivity for IgA isotype anti-GP2 antibody (Log-rank p=0.008) predicted OLTx and/or liver-related death. Accordingly, univariate Cox regression analysis revealed anti-GP2 IgA-positivity as a predictor for poor disease outcome (HR: 5.15 [1.33–19.97], p=0.018), that remained an independent predictor after adjusting for Mayo risk score in multivariate Cox-regression analysis (HR: 4.69 [1.05–21.04], p=0.043). A similar tendency was found when we adjusted for the presence of cirrhosis, instead of Mayo risk score (HR: 3.74 [0.9–15.55], p=0.07). In subpopulation analyses, positivity for

anti-GP2 IgA was associated with poor outcome in the subgroup of patients with adult onset PSC (Log-rank p =0.034) and concomitant IBD (Log-rank p<0.001), but not in the subgroup of patients with pediatric onset PSC (Log-rank p=0.098) and no concomitant IBD (Log-rank p=0.666).

Anti-GP2 IgA antibody status is associated with increased secretory IgA concentration

Serum levels of total sIgA were significantly higher in patients with PSC compared to healthy controls (median [IQR], 96 [73–180] vs. 30 [21–42] μg/mL, p<0.001). Furthermore, anti-GP2 IgA positive cases had higher sIgA levels compared to anti-GP2 IgA negative ones (149 [86–246] vs. 89 [71–118] μg/mL, p=0.021).

Characterization of GP2 antibodies

Finally, we characterized anti-GP2 IgA antibodies in patients with PSC and Crohn's disease in a flow-cytometry subtyping assay. The presence of secretory component (SC) on GP2 IgA antibodies was 68.4% in PSC (13/19) similar to the frequency (75%, 9/12) observed in patients with CD without PSC. In both PSC and CD, IgA1 was the dominant isotype. However, in patients with CD, the median IgA2 ratio was only 2.02% (IQR: 1.53–2.47%), while in patients with PSC, this was 6.75% (IQR: 4.19-9.79%).

DISSCUSSION

Both reduced and increased serum ferritin levels may indicate pathological processes in certain groups of cirrhotic patients

In a large cohort of cirrhotic outpatients, we measured serum ferritin levels and evaluated its association with the development of disease-specific complications and mortality during a 2-year-long follow-up period. We found that ferritin's serum concentration was significantly higher in males and older patients, as it was previously reported. We also observed significantly lower levels in patients, who previously had variceal bleeding while anemia was not associated with such difference in these patients.

However, when we investigated correlations of ferritin with other laboratory measures, we found that parameters associated with hematopoiesis and iron homeostasis (hemoglobin, hematocrit, mean corpuscular hemoglobin and mean corpuscular volume) correlated with ferritin in both compensated and decompensated groups, while inflammation markers and disease severity measures did only in the latter group. This result indicates that although in advanced stages of cirrhosis, inflammation and disease severity are important

modulators of ferritin level, in earlier stages, ferritin concentration mainly corresponds to the iron homeostasis of the body, much like in the healthy population. Previous studies reported that CAID predominantly manifests in decompensated stage as a result of SIRS and CARS. This observation is in line with the lack of correlation between ferritin level and inflammatory measures in our compensated outpatients.

Aalan-Johansen and Kaplan-Meier analyses revealed that compensated patients with low serum ferritin levels had the highest incidence of decompensation during follow-up, while decompensated patients with high ferritin levels had the highest incidence of BI development. Mortality was associated with high ferritin level in every patient group. Previous studies reported association only between high ferritin levels and worse outcomes, however these investigations were conducted involving patients with ongoing AD. Our observation that low serum ferritin concentration is associated with an increased risk of developing a decompensated clinical stage in patients with previously compensated cirrhosis is definitely an interesting and unexpected novel finding. Hence, some speculation is needed in order to interpret these results. Based on the observed correlations, we assume that compensated patients, who did not have previous acute bleeding but had low ferritin levels might have had chronic, subclinical (unrecognized) gastrointestinal hemorrhage leading to depleted iron stores and low ferritin concentration. In cirrhotic patients, this form of bleeding is mostly due to increased portal pressure (reaching the clinically significant threshold), which is a well characterized risk factor of AD. When we checked gastroscopy results of patients who had low ferritin levels and developed decompensated clinical stage during follow-up, we found that 7 out of 8 patients had esophageal varices and/or portal hypertensive gastropathy at time of recruitment. This observation provides evidence for the presence of increased portal pressure in these patients. Consistently, in multivariate Cox regression, besides low ferritin level, anemia was also an independent predictor of the development of first AD events, further supporting the hypothesized connection between hidden blood loss and decompensation. However, the presence of chronic bleeding still needs to be verified by specific methods, for instance, fecal blood test in future studies. With that being said, ferritin measurement seems to have the potential to provide an important additional screening tool in the evaluation of both early and advanced stage cirrhosis, aiding the appropriate clinical decisions in the future.

On the other hand, since low ferritin level was associated with higher incidence of decompensation, it might seem controversial that mortality was associated with higher ferritin levels in the compensated population. As discussed, elevated ferritin level indicates either iron overload, which is toxic

to cells, or increased inflammation, caused by either bacterial translocation or a subclinical starting infection. These last two, in return, can lead to overt infections, while inflammation in general and iron-induced toxicity can trigger the progression of HCC and comorbidities ultimately leading to increased overall mortality. Indeed, almost every patient with high ferritin level who developed decompensated clinical stage died, and some deceased even without having an AD episode. In contrast, no patient with low ferritin level who did not develop decompensated clinical stage died during follow up. Furthermore, approximately 40% of those with low ferritin levels who did decompensate, also survived. The explanation for this difference is not clear, but the decompensation of non-inflammatory mechanisms, indicated by lower ferritin levels, might represent a less severe/more manageable type of AD.

Since routine laboratory methods are designed to measure L-ferritin, we do not have information about the ratio of H-ferritin or iron saturation of serum ferritin, which could expand our understanding of the underlying processes. Additionally, transferrin level and saturation as well as liable iron level could have further supported our hypothesis about how low ferritin level is connected to decompensation. The lack of these data is a limitation of the present study and must be assessed by further investigations; however, the association of low ferritin level and low iron storage is evident even without the aforementioned laboratory results.

Finally, in decompensated patients, high ferritin level was associated with both investigated outcomes (BI and mortality). Yet, in this subpopulation, ferritin level lost statistical significance in the multivariate model of mortality (i.e., could not predict the outcome independently from other factors involved in the model). This observation may be explained by the detected correlations between ferritin level, inflammatory markers and disease severity scores in these patients, as similar correlations were not seen in the compensated subpopulation.

In summary, both low and high ferritin concentrations are more detrimental than normal levels, but for different hypothesized reasons. Both indicate distinct pathological processes playing roles in the development of different adverse outcomes. In cirrhotic patients who did not have previous acute bleeding but have low ferritin levels, the physician should consider looking for occult blood loss and signs of increasing portal hypertension, while all patients with high ferritin levels are subject to increased mortality risk and require closer follow-up or intervention. These novel results need further exploration, but they are of importance and may support the need for including ferritin among markers routinely used to follow disease progression of patients with cirrhosis.

Measurement of serum sTREM-1 levels can assist diagnosis of cirrhosis associated BIs and predicting short-term mortality

To the best of our knowledge, this is the first study, reporting the feasibility and the usefulness of sTREM-1 for the diagnosis and prognosis of cirrhosis-associated BI in clinical settings. First, we evaluated the association between sTREM-1 levels and disease-specific characteristics of our cirrhotic patients. We showed that in the total cohort, sTREM-1 concentration displayed a gradual increase according to disease severity (Child-score). However, subdividing cases to patients with and without AD revealed that in the absence of AD sTREM-1 levels are not significantly higher in chronically decompensated patients. Furthermore, AD cases without BI showed increased sTREM-1 levels only compared to compensated clinical stage or Child-Pugh A severity outpatient groups. AD patients with BI, however, had highly elevated sTREM-1 serum concentrations. Similarly, paired samples from patients in their clinically stable and AD state showed significantly different sTREM-1 levels only if BI was also present. However, we detected significant correlations between sTREM-1 levels and disease severity scores, especially in the AD group where association of sTREM-1 concentration with CLIF-AD score was the most remarkable. Based on these results, sTREM-1 concentration is tightly associated with infection in cirrhotic patients and affected by disease state only to a lesser extent. Furthermore, there is a clear association between sTREM-1 level and severity of infection since septic patients had even higher serum concentration of this marker. This result is also supported by strong correlation of sTREM-1 levels with white blood cell and neutrophil counts as well as CRP, known to be significantly elevated during BIs.

Next, we evaluated capacity of sTREM-1 to diagnose BIs in cirrhotic patients in comparison with CRP. sTREM-1 and CRP are both considered as nonspecific inflammatory markers but of different cellular origin. CRP is synthesized by the liver, therefore the release of the molecule depends on the liver function. In fact, a previous study showed that the more severe the underlying liver dysfunction, the lower the CRP response to bacteremia was. Similarly, it was reported that the accuracy of CRP for diagnosing BI decreased in advanced stage of cirrhosis or in presence of ascites. While in low activity liver disease CRP could indicate BIs at a level of >10 mg/L, in the case of advanced liver disease patients this cut off was >30 mg/L, which is corresponding to our findings (>9.1 mg/L and >36.2 mg/L). In contrast, sTREM-1 is mainly produced by neutrophils, monocytes and macrophages, although it was reported to also be inducible in endothelial cells. Therefore, it seemed possible that sTREM-1 might be a better screening marker for BIs in cirrhosis. To address this hypothesis, we performed ROC analyses of AD

patients with BI vs. outpatients or AD patients without BI. As expected, CRP showed considerably reduced performance in the second scenario, but sTREM-1 also provided similar discriminative power to CRP in both cases. However, the absolute reduction in accuracy of CRP was greater than the one observed in the case of sTREM-1, which corresponds to our hypothesis about the effect of the cellular origin.

Several other biomarkers have been tested for infection in cirrhosis so far but no markers could exceed the clinical efficacy of CRP for detecting the presence of BI in cirrhotic patients except procalcitonin and presepsin, but they showed to be superior exclusively in severe infections. A recent approach in laboratory medicine is the generation of composite scores from individual markers that clearly exceed the efficacy of the separate parameters. The base of these approaches is the measurement of individual analytes using single or multiplex assays. The next step is to fuse these tests into one composite parameter using modern mathematical approaches like principal component, random forest analysis, logistic regression, or other sophisticated mathematical algorithms. In this study we followed this line and created a composite score - using logistic regression model - from CRP and sTREM-1 in combination with a clinical parameter (presence of ascites). Ascites is a sign of decompensated liver function, thus it can correct the curve where the investigated markers are expected to underperform. Additionally, it is also a well-known risk factor of BI in cirrhotic patients. Adding the best discriminative cut-off of CRP to the natural logarithm of sTREM-1, also taking presence of moderate/severe ascites into account, resulted in a significantly more powerful score compared to the individual parameters. Therefore, our results further support the concept that fusion of individual tests into one parameter can create a superior marker for a certain clinical situation. While practically no marker on its own could exceed the efficacy of CRP in identification of cirrhosis associated BI so far, our composite score had a significantly better performance.

Another suitable approach for combining individual tests is simply adding points if any of the markers are above their established cut-off levels, which might be even easier to use in clinical practice. This method obviously does not result in a continuous scale, thus its AUCROC cannot be compared to the original markers by ROC curve analysis, however the associated overall accuracy, sensitivity, specificity, positive and negative predictive values and likelihood ratios can be calculated. Additionally, we created this score as well and showed its parameters in comparison to the individual markers and the ADI score. While the two composite parameters are very comparable, ADI performs slightly better in finding patients with BI (sensitivity: 76.8% vs. 71.4%). However, the simplicity of the “at least one positive marker” method

and the fact that it does not contain the somewhat subjective "presence of ascites" parameter may overweight this difference in clinical practice. Another point to be considered in the case of composite scores is the increase in the diagnostic costs, but this must be weighted by the clinical advantage provided by the new parameter.

Finally, we aimed to assess whether sTREM-1 can provide prognostic information in cirrhosis associated BIs. In patients with BI, increased sTREM-1 level was significantly associated with higher 90-day mortality in both Kaplan-Meier and univariate Cox regression analyses. In multivariate model, high sTREM-1 concentration remained an independent risk factor of 90-day mortality of patients with BI besides ACLF grade. Notably, ln(sTREM-1) also overperformed MELD and CLIF-AD scores in our cohort. Previous reports showed that procalcitonin and sCD163 were risk factors of mortality in cirrhotic patients with BI while presepsin was not. Based on the HR values and even more on the Kaplan-Meier analyses sTREM-1 seems to have the highest predictive power of these markers concerning the mortality of the patients: after 90 days about 80% of the patients with AD+BI were alive if the serum sTREM-1 concentration was below 660 pg/mL, while only 25% if the sTREM-1 was above this cut-off. These values were about 85% and 50% for sCD163 using a cut-off of 7000 ng/mL.

Importantly, sTREM-1 values also showed association with the presence of organ injury (AKI) and failure (ACLF), which are common complications of BI in cirrhotic patients. Notably, since sTREM-1 is a 15 kDa molecule, renal impairment can also directly contribute to elevation of sTREM-1 levels due to reduced excretion of sTREM-1 from the circulation via the kidneys. Therefore sTREM-1 based diagnosis of BI might require more caution in patients with AKI. However, AKI could primarily increase the false positive ratio, overestimating the number of patients with BI, but this is more acceptable clinically than the other way around. Interestingly, this effect was not observed in our cohort, since cirrhotic patients without BI, had no significant difference in their sTREM-1 concentrations in the presence or absence of AKI (although there were only 5 individuals in the former group). Furthermore, the cause for developing AKI in the first place is often BI itself in cirrhotic patients, therefore the impact of kidney function on the diagnostic accuracy of the marker for BI might not even be significant anyway. Similarly, since kidney injury itself is a well-known risk factor of mortality, we argue that the presence of AKI does not disqualify high sTREM-1 level to be used for mortality prediction. Especially since high sTREM-1 concentration could predict 90-day mortality independently from ACLF, in which kidney failure was found to be the most important factor in terms of mortality. However, another study investigating the applicability of sTREM-1

in cirrhotic patients with AKI for the diagnosis of BI would be needed to accurately access these questions.

sTREM-1 cut-offs encompass a wide range in different studies. In our work 429 and 642 pg/mL were the best discriminative values for the diagnosis of BI in cirrhotic patients. Although, in most reports, cut-off levels are lower than ours, it can be explained by several factors. It is important to emphasize that our controls were cirrhotic patients and not healthy individuals. As sTREM-1 concentration increases in some extent parallel with the severity of liver disease, it could push the discriminative threshold towards higher values. Furthermore, serum sTREM-1 measurement requires careful sample handling, since it has been reported to be sensitive for repeated freeze/thaw cycles. Therefore, we measured this marker from aliquots we thawed for the first time to avoid its degradation. Finally, several assays are available and used in different studies that may provide different sTREM-1 concentrations in the tested samples.

One of the limitations of the present study is indisputably that serum sTREM-1 concentration was only measured at enrolment, and thus dynamic changes of its level were not assessed. Validation of our results will be also needed to prove their general adequacy. For these reasons, additional clinical studies will be needed to further investigate sTREM-1 concentrations including serial changes and their possible association with different outcomes during infection.

The present study suggests that sTREM-1 is a promising biomarker during diagnostic procedure of BIs in cirrhosis. We found performance of sTREM-1 to be similar to CRP in these clinical settings. However, combination of these markers with the presence of ascites provides a significantly more accurate diagnostic work-up for BI in cirrhosis. Furthermore, we also demonstrated that high sTREM-1 level is an independent predictor of infection-related short-term mortality in these patients.

Anti-GP2 IgA autoantibodies are specific markers of disease progression in PSC and provide evidence for the significance of intestinal-liver interactions

Previously, our study group observed high frequency of PSC in anti-GP2-positive CD patients. We, and others reported associations between anti-GP2 antibodies and various aspects of progressive disease course in CD patients. Interestingly only IgA, but not IgG isotype antibodies were related to the development of complications in that prospective, large-scale IBD study. Therefore, we aimed to investigate the prevalence and clinical importance of

ant-GP2 antibodies in a prospective PSC cohort with respect to different immunoglobulin isotypes.

Simultaneously with a research group from Germany, we demonstrated enhanced IgA type anti-GP2 antibody formation in PSC. We used the same cell-based IIF test with an identical cut-off value. As shown elsewhere, IIF test appears to detect anti-GP2 IgA in patients with PSC better in contrast to ELISA. The IIF test used in this study detects the interaction of IgA with glycosylphosphatidylinositol (GPI)-anchored GP2 on the surface of HEK239 cells. This might provide a unique epitope structure of GP2 which could be different from one of GP2 adsorbed to ELISA solid phases. A careful comparison of our results with the findings of *Jendrek et al*. revealed however, some differences. In our PSC patient cohort, occurrence of anti-GP2 IgA was lower (30.8% vs. 48.7%),. The reason for these differences is not fully understood, but is most probably attributed to the composition of the patient populations. First, the Mayo risk score reflecting disease severity was higher in their cohort. Second, the occurrence of biliary tract cancer was 12.3%, while none of our patients had CCA at baseline. Third, in that study anti-GP2 IgA positivity was not exclusively associated with PSC, but rather with the presence of large bile duct diseases, irrespectively of their malignant or benign character based on findings in their disease control groups. Finally, in our study, we used an extensive CLD group with various etiology as disease controls in contrast to the study of *Jendrek et al*. Neither patients with small bile duct disease (PBC), nor patients with alcoholic liver cirrhosis showed increased anti-GP2 IgA frequency. This latter finding is particularly intriguing, because in cirrhosis we found high frequency of various serological antibodies, mainly of IgA isotype, such as ASCA (38.5%) or ANCA (52.2%) that are also frequent in PSC.

Exploring patient characteristics at enrolment, the presence of anti-GP2 IgA-positivity was associated with a more severe disease phenotype. Consistently, anti-GP2 IgA predicted faster disease progression during follow-up, even after adjusting for the Mayo risk score or the presence of cirrhosis. In our study, both liver-related death and OLTx were considered as equal endpoints, since they represent the development of end-stage liver disease as the result of progressive fibrosis. In the parallel study of *Jendrek et al*., anti-GP2 IgA-positivity also identified a subgroup of patients with high mortality. Notably, poor survival was primarily attributed to cholangiocarcinoma (CCA) and not to the development of end-stage liver disease in their cohort. In our cohort, only 1 patient (1.5%) developed biliary tract cancer during follow-up, therefore evaluation of anti-GP2 IgA regarding the development of CCA was not possible. This CCA rate is lower than the reported 8–13.2% in some other studies, but equals with those ones from Israel (2.1%) and the Netherlands

(3.6%) with corresponding follow-up times in recent reports. Supporting clinical evidence for the anti-GP2 IgA - fibrosis linkage in PSC might be the observation that anti-GP2 IgA was more prevalent in CD patients with stenotic disease in previous reports.

This idea is further supported by the results of another paper in which the presence of IgA autoantibodies against isoform 1 and 4 of the GP2 protein was identified as a risk factor for associating cirrhosis and more severe disease phenotype in PSC. Furthermore, according to a recently accepted study, antibodies specific for isoform 1 were associated with shorter transplant-free survival, while antibodies recognizing isoform 4 were independent risk factors for the development of CCA in two independent patient populations. All of these provide sufficient evidence for the usefulness of integrating anti-GP2 IgA antibodies into clinical practice, offering a solution to a long-standing and unresolved clinical problem for the first time.

To explore the possible link between mucosal immunity and the development of severe phenotype in PSC, the serum levels of total sIgA were measured. A novel finding of our study is that in PSC, the total sIgA levels were significantly elevated, namely three-fold higher, compared to healthy controls. These results indicate an elevated retro-transport of sIgA from the gut mucosal surface in PSC. An additional two-fold elevation of total sIgA levels were found in anti-GP2 IgA-positive cases. These results suggest that retro-transport is specifically further enhanced in patients with anti-GP2 IgA-positivity. Flow-cytometry based characterization of anti-GP2 IgA-positive samples revealed that SC was present on these molecules in up to 68.4% of PSC cases. At the same time, the proportion of IgA2 isotype anti-GP2 did not increase above 10% that is still considered normal in the healthy population. This may be due to the protein nature of the GP2 antigen, as a previous study found that protein antigens are much weaker inducers of IgA2 production than polysaccharides. On the other hand the high percentage of SC strongly indicates that after secretion to the gut lumen, anti-GP2 IgA antibodies are retro-transported across the mucosal epithelium. Retro-transported sIgA molecules, however, are not “lonely” particles, but rather exist in bounding with their antigens. In this way, enhanced retro-transport of anti-GP2 IgA might increase microbial overload in the mucosal compartment and perpetuate antigen-induced signaling. Memory T lymphocytes primed in the inflamed gut and homing to the biliary tract via aberrantly expressed adhesion molecules plays a fundamental role in the extension of gut inflammation to the biliary tract. Interestingly, FimH has been recently identified as a ligand of Toll-like receptor (TLR)-4. Sustained TLR4 activation leads to enhanced fibrosis through TGF-beta signaling. At the same time, FimH elicits an immune response with enhanced type I Interferon production that has been linked to

disease amplification in autoimmunity. These mechanisms could serve an explanation of how the breakdown of tolerance towards GP2 in the gut is associated with the development of enhanced fibrosis, and thus disease progression in the liver. The GP2—FimH axis deserves further exploration in the pathogenesis of PSC and might also be a highly intriguing issue from the therapeutic point of view. GP2 has a high structural and functional homology with uromodulin (Tamm-Horsfall protein in the urinary tract). Recombinant vaccine against the adhesion protein of FimH is already under development in recurrent urinary tract infections. Furthermore mannose-derived FimH antagonists, also hold promise as novel treatments for UTIs and Crohn's disease.

In summary, the presence of anti-GP2 IgA antibodies is associated with a more severe PSC phenotype, but not with other CLDs including cholestatic liver disease with small bile duct involvement. The findings of our prospective referral cohort study indicate that anti-GP2 IgA may be a useful additional serological tool in the stratification of PSC patients and is associated with the progressive disease course. In addition, occurrence of IgA type anti-GP2 antibody may serve as an additional clue towards the significance of gut-liver interactions in the disease course of PSC.

Thus, using modern laboratory and statistical approaches, we identified and characterized 3 promising markers for the management of chronic liver disease, and explored certain mechanisms of the underlying processes. These markers have diagnostic as well as short- and long-term prognostic potential, and although further research is needed, they may help clinicians in the future to provide these patients with the most appropriate treatment available.

New results and clinical relevance of the PhD thesis

Serum ferritin level is an independent predictor of

1. the development of a decompensated clinical stage in patients with compensated cirrhosis (if serum ferritin is <40 μg/L);
2. bacterial infections in decompensated cirrhotic patients (if serum ferritin is >310 μg/L);
3. mortality in the entire outpatient population (if serum ferritin is >310 μg/L) during a 2-year-long follow-up.

4. The diagnostic efficacy of sTREM-1 in identifying bacterial infections in patients with cirrhosis is similar to that of CRP.
5. The combination of sTREM-1, CRP and the presence of ascites into a composite score significantly increases the diagnostic power of the individual molecules.
6. sTREM-1 level of more than 660 pg/mL is an independent risk factor for 90-day mortality in acutely decompensated cirrhotic patients with bacterial infection.

Presence of anti-GP2 IgA antibodies in PSC:

7. is associated with a more severe form of the disease;
8. is an independent predictor of shorter transplant-free survival even after correction for Mayo risk score.

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Registry number: DEENK/96/2021.PL
Subject: PhD Publication List

Candidate: Dávid Tornai
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Tornai, D.**, Antal-Szalmás, P., Tornai, T. I., Papp, M., Tornai, I., Sipeki, N., Janka, T., Balogh, B., Vitális, Z.: Abnormal ferritin levels predict development of poor outcomes in cirrhotic outpatients: a cohort study.
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4. **Tornai, D.**, Papp, M.: Editorial: serologic antibodies in primary sclerosing cholangitis - a tell-tale sign of compromised gut-liver immunity?
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IF: 2.837

Total IF of journals (all publications): 88,621
Total IF of journals (publications related to the dissertation): 9,218

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

11 March, 2021

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