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

THE ROLE OF CLINICAL, SEROLOGICAL AND GENETIC FACTORS IN THE PREDICTION OF BACTERIAL INFECTIONS AND TRANSLOCATION IN PATIENTS WITH LIVER CIRRHOSIS

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The Examination takes place at Institute of Surgery, Faculty of Medicine, University of Debrecen, on the 12th of July 2021 at 10.00 (online).

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Publicity is guaranteed during the online Defense. If you are willing to participate, please indicate via email to dinya.tamas@med.unideb.hu until 4.00PM on the 11th of July 2021

ABBREVIATIONS

ACLF: Acute-on Chronic Liver Failure
AD: Acute Decompensation
AIH: Autoimmune Hepatitis
AMA: Anti-Mitochondrial Antibody
APP: Acute Phase Protein
ASCA: Anti-*Saccharomyces cerevisiae* Antibody
AMCA: Anti-*Mannobioside* Antibody
ALCA: Anti-*Laminaribioside* Antibody
ACCA: Anti-*Chitobioside* Antibody
BT: Bacterial Translocation
BactDNA: Bacterial DNA
BPI: Bactericidal Permeability-Increasing Protein
CAID: Cirrhosis-Associated Immunodeficiency Syndrome
CI: Confidence Interval
CLD: Chronic Liver Disease
CP: Cumulative Probability
CRP: C-Reactive Protein
DNA: Deoxyribonucleic Acid
EndoCab: Endotoxin Core Antibody
ELISA: Enzyme-Linked Immunosorbent Assay
ERCP: Endoscopic Retrograde Cholangiopancreatography
FRET: Fluorescence Resonance Energy Transfer
FCN: Ficolin
GBP: Gram-positive
GNB: Gram-negative
HCC: Hepatocellular carcinoma
HCV: Chronic Hepatitis C
HR: Hazard Ratio
IBD: Inflammatory Bowel Disease
IL: Interleukin
IQR: Interquartile Range
kDa: Kilodalton
LBP: Lipopolysaccharide-Binding Protein
LPS: Lipopolysaccharide
MBL: Mannose-Binding Lectin
MCP-1: Monocyte Chemoattractant Protein-1
MELD: Model for End-Stage Liver Disease
MRCP: Magnetic Resonance Cholangiopancreatography
NAFLD: Non-Alcoholic Fatty Liver Disease
NDP52: Nuclear Dot Protein 52
NOD2: Nucleotide-Binding Oligomerization Domain-Containing Protein 2
NSBB: Non-Selective Beta-Blocker
OR: Odds Ratio
PBC: Primary biliary Cirrhosis
PCT: Procalcitonin
PCR: Polymerase Chain Reaction
PPI: Proton Pump Inhibitor
PPV: Positive Predictive Value
PSC: Primary Sclerosing Cholangitis
RNS: Ribonucleic Acid
PRR: Pattern Recognition Receptor protein
SBP: Spontaneous Bacterial Peritonitis
SE: Standard Error
SIRS: Systemic Inflammatory Response Syndrome
SNP: Single Nucleotide Polymorphism
sPRM: Soluble Pattern Recognition Molecule
TNF-α: Tumor Necrosis Factor Alpha
TLR: Toll-Like Receptor

ABSTRACT

***Background**:* Development of bacterial infections – often in their more severe clinical forms – is frequently seen in liver cirrhosis. Infections make the liver disease worse by impairing hepatic function, provoking the onset of complications (e.g. coagulopathy, hepatorenal syndrome, hepatic encephalopathy, variceal bleeding, etc.), and are themselves important factors of mortality. The role of cirrhosis-associated immune dysfunction (CAID) syndrome and pathologic bacterial translocation (BT) in the development of infections is significant, and even more profound in more advanced disease stages. Reliable prediction of impending infections is necessary to be able to manage complications, slow disease progression and decrease mortality. The functional polymorphisms of pattern recognition receptors (PRR) affect their ability to detect and eliminate pathogens, thus impairing innate host defense mechanisms. The role of innate PRR-related immune deficiencies in liver cirrhosis is confirmed in spontaneous bacterial peritonitis (SBP). However, neither their impact on non-SBP type bacterial infections, nor their significance in disease progression has not been thoroughly investigated. The antibody production against surface carbohydrate and other protein antigens of gut bacteria is a well-known serological process in complicated intestinal Crohn's disease, and due to its connection to NOD2/CARD15 mutations, it is considered to arise – at least partially – from a genetic loss of tolerance against the gut flora. We hypothesized that the production of IgA isotype antimicrobial antibodies is not disease specific, it can be acquired, and it can predict the onset of pathologic BT. The prevalence of these antibodies in liver cirrhosis and their predictive value for the development of bacterial infections has not been investigated so far.

Aims: Within the framework of a large, prospective observational study of patients with cirrhosis, we investigated the role of known and clinically significant genetic polymorphisms of certain surface and intracellular pattern recognition receptor proteins (PRRs) of the innate immune system (toll-like receptors [TLR2 and 4] and NOD2/CARD15) in the development of bacterial infections and disease progression (decompensation and/or liver-related mortality). We also examined their connection with the known serological markers of endotoxin exposition (Lipopolysaccharide-Binding Protein [LBP] and endotoxin core antibody [EndoCab]). We determined the frequency of several isotypes of IgA antimicrobial antibodies (ASCA, anti-OMP Plus™ IgA), their correlation with the clinical manifestation and the various PRR polymorphisms. We determined the clinical significance of the serological antibodies in the development of bacterial infections and infection-related mortality. The control group consisted of a large number of patients with inflammatory bowel disease (IBD), non-cirrhotic chronic liver disease, and healthy individuals.

***Methods**:* Evaluation of antimicrobial antibodies and biomarkers of endotoxemia was done from serum samples using ELISA assays. Three alleles of NOD2 gene (R702W, G908R és L1007PfsinsC), and 1-1 allele of the *TLR2* (−16934T>A) and *TLR4* (D299G) genes was genotyped.

***Results**:* After investigating the risk factors of bacterial infections in patients with liver cirrhosis, from generic factors representing innate susceptibility only *NOD2* gene variants proved to be risk factors of SBP, while *TLR2* or *TLR4* alleles did not. In ascitic patients the cumulative probability of SBP development was higher in the presence of *NOD2* gene variants, as compared to the wild type. (76.9±19.9% vs. 30.9±6.9%, *pLogRank*=0.047). At the same time, regarding the development of non-SBP type infections, the clinical factors proved to be more significant and did not show correlation either to the individual, or the combined PRR gene profiles. Advanced disease stage (HR [95% CI]: 2.11 [1.38-3.25]) and history of a prior infectious episode (HR: 2.42 [1.58-3.72]) was the most direct clinical risk factor for the development of a subsequent bacterial infection. The risk of non-SBP type bacterial infections was even higher when both clinical factors were present (HR: 4.74 [2.68-8.39]). The PRR genotypes were also not able to predict the long-term disease outcome in liver cirrhosis.

The production of various antimicrobial antibodies in liver cirrhosis was more frequent (ASCA IgA/IgG: 38.5% and anti-OMP Plus™ IgA: 62.6%) compared to both the healthy controls (16% and 20%, *p< 0.001* for both), and to the chronic liver patient control group (22.2% and 16.5%, *p<0.001* for both). Their formation was more likely determined by acquired factors, like disease severity, than genetic factors predisposing to BT, like *NOD2* gene variants. From the ASCA antibodies the IgA isotype proved to be an independent risk factor of bacterial infections, regardless of disease severity (HR: 2.18 [1.31-3.61]).

Conclusions*:* We could confirm that certain frequent *NOD2* alleles increase the risk of SBP. However, the various polymorphisms of *NOD2* and *TLR2*/*4* did not affect the development of non-SBP type bacterial infections. History of a previous infectious episode was confirmed to be a significant, novel risk factor - regardless of disease severity – for subsequent infections. The investigated PRR alleles did not correlate either with the serological markers of BT, or with the occurrence of clinical complications during the follow-up period, or with liver-related mortality. These results suggest that the PRR genotype in cirrhosis has only a limited predictive value for the course of disease. The presence of IgA isotype ASCA antimicrobial antibodies is frequent in liver cirrhosis, and can be considered a novel serological marker of pathologic BT. The IgA isotype of ASCA antibodies are new serological risk factors of cirrhosis-related bacterial infections.

INTRODUCTION

Epidemiology and progression of cirrhosis

Liver cirrhosis is an irreversible process developing on the ground of chronic diseases, during which inflammation, necrosis and then scarring occur in the parenchyma of the liver owing to various mechanisms. Due to the regenerative ability of the liver, hepatic functions remain undisturbed for a while (compensated phase), then they gradually begin to deteriorate (decompensated phase) until the onset of liver failure. Liver cirrhosis can be caused by several factors, from which in Europe the most common ones are the excessive alcohol consumption and the non-alcoholic fatty liver disease (NAFLD). Cirrhosis lays an increasing burden on the health care system in terms of morbidity and mortality rates. It is the fourteenth most common cause of death worldwide, while fourth in Central-Europe. The prevalence of cirrhosis is difficult to assess and is probably higher than reported, because the initial stages - sometimes even the decompensated stage - may remain asymptomatic. Previously, cirrhosis was considered an end-stage disease leading inevitably to death unless the patient could undergo liver transplantation. Lately, this perception has entirely changed and today the disease is more likely considered an alterable, dynamic process. One-year mortality varies from 1% to 57% depending on the stage of cirrhosis and complications associated with decompensation episodes are considered to be the major risk factors of mortality. In cirrhosis, impairment (so-called acute decompensation [AD]) can develop basically in any stage of the disease suddenly, in days or weeks, accelerating the progression and/or leading to death. Further worsening during the decompensation episode leads to acute-on-chronic liver failure (ACLF) which has very high short-term mortality (> 50%) and may result in one or more extrahepatic organ failure (liver, kidneys, brain, lungs or vascular). Acute disease progression is usually caused by some sudden impact, like in most cases bacterial infection.

Bacterial infections

Cirrhosis is the most common acquired immunodeficiency disorder. Not only are these patients particularly susceptible to bacterial infections – and often in their more severe forms -, but also these episodes trigger the aggravation of the liver disease. A wide range of bacterial infections are able to impair hepatic function, both directly and indirectly through inflammatory mediators (systemic inflammatory response syndrome, SIRS), and infections can induce a variety of complications in cirrhosis such as coagulation disorders, hepatorenal syndrome, hepatic encephalopathy, variceal bleeding and may lead to death. These is especially true if the infection results in ACLF. In the presence of bacterial infection, mortality increases approximately fourfold, regardless of the severity of cirrhosis. Thirty percent of patients die within 1 month of admission and another 30% die within a year after the onset of infection. Moreover, patients with cirrhosis die twice as often from sepsis than patients without cirrhosis. Recent literature data suggest that the development of bacterial infections represents a distinct prognostic stage independent of disease severity and alters the course of the disease.

During hospitalization and treatment of patients with cirrhosis, some sort of infection was detected in 32-34% of patients. Bacterial infection is more likely to develop in the presence of more severe liver disease (Child-Pugh C), gastrointestinal bleeding or diabetes. During hospitalization for gastrointestinal bleeding, the incidence of bacterial infections is even higher, at about 45%. The frequent occurrence of intestinal pathogens is the characteristic feature of bacterial infections in cirrhosis. The most common infection in patients with ascites is spontaneous bacterial peritonitis (SBP), but various non-SPB type infections, such as pneumonia or urinary tract infection also occur. If a cirrhotic patient has already undergone SBP, secondary antibiotic prevention is recommended to avoid the recurrence of the disease. However, the risk-increasing effect of non-SBP type infections as the source recurrent bacterial infections has not been studied yet, and post-infection prevention strategies are still unknown.

In cirrhosis, the immune system is disrupted at several points. Cirrhosis-associated immune dysfunction (CAID) affects both innate and adaptive components of the immune system's response to microbial challenges, since not only local, but systemic immune functions of the liver are impaired. Bacterial filter function of the liver is impaired due to the diminished reticulo-endothelial system function and the reduced number of Kupffer cells. As a result, this structural derangement reduces the clearance of bacteria translocated from the gut and of such bacterial products such as endotoxins. The presence of portosystemic shunts further enhances the direct entry of bacteria and bacterial products into the systemic circulation. Due to the uncontrolled bacteremia, the immune system is in a constant activated state. As a result of the decreased synthetic liver function, the amount of proteins produced in the liver including innate immune proteins, the soluble pattern recognition receptor proteins (PRR) and the acute phase proteins (APP) is also insufficient leading to manifest immunodeficiency. CAID is a dynamically changing phenomenon that includes both systemic inflammatory responses and immunodeficiency. It may be associated with immune paralysis, characterized by an increase in the level of anti-inflammatory cytokines and a decrease in the level of pro-inflammatory cytokines. Furthermore, structural and functional damage induced by intestinal inflammation, decreased mucosal defense mechanisms and altered intestinal flora together lead to abnormal bacterial translocation.

Abnormal BT is characteristic primarily in the advanced stage of cirrhosis and plays an important role in the pathogenesis of the disease and the development of various complications. Its direct clinical consequences are the development of SBP and bacteremia. Besides SBP, other systemic infections may be associated with BT, but evidence on this is not yet clear. Even in the absence of an overt bacterial infection, sustained entry of various bacterial products into the hepato-splanchnic and systemic circulation can also have a detrimental effect by inducing an enhanced pro-inflammatory response. Failure to control invading bacteria and/or their products - together with an increased host susceptibility to infection -, may result in the damage of remote organs. The development of consecutive organ failure(s) is a significant mortality risk factor in this patient cohort.

The major impact of BT on the pathogenesis of the disease is confirmed by evidence that selective intestinal decontamination with oral antibiotics reduces the overall risk of infections and improves short-term survival in high-risk individuals. In the absence of overt infections, norfloxacin also normalizes elevated proinflammatory cytokine levels and increases vascular resistance in patients with ascites who present with a high lipopolysaccharide-binding protein (LBP) level. The development of bacterial resistance as an unwanted consequence of antibiotic prophylaxis, however, means an emerging problem.

Possibilities to predict bacterial infections

Accurate detection and risk assessment of the development of abnormal BT in cirrhosis can support the effectiveness of treatment strategies against bacterial infections and other complications. Unfortunately, reliable and specific serological methods are not yet available in everyday clinical practice to detect the presence and severity of abnormal BT. Recently, certain biomarkers detected in the serum, plasma, ascites or stool have been confirmed to be markers of long-term exposure to the intestinal flora. However, it has not yet been proven which sampling source is the most optimal for each biomarker. Similarly, we could not establish the threshold values above which the level of a given biomarker certainly reflects pathological BT. Although the number of promising biomarkers – and also the number of scientific publications investigating their physiological role - is constantly increasing, we still miss that one highly specific and thoroughly validated laboratory marker which could surely predict the development of bacterial BT in liver cirrhosis and its impact on the course of disease.

The lipopolysaccharides (LPS) or endotoxins, the main outer membrane components of Gram-negative bacteria, were the first discovered serological markers of bacterial translocation; however, they are hardly used anymore. LPS has a very short half-life (2-3 h) and its level is influenced by many factors resulting in a low sensitivity. LBP has a significantly longer half-life and thus it is considered a relatively reliable marker for the diagnosis of BT. LBP is produced by hepatocytes in response to bacteremia and endotoxemia. The main limitation of serum level measurement of LBP in clinical practice is the high cost of the examination. Therefore, due to the complexity and high cost of the method, it is currently not used routinely. Bacterial DNA (BactDNA) is a new assay marker in BT. BactDNA appears to be a better marker than endotoxin assay as it can be detected in serum for a longer period (1-3 days) and may be associated with an immune activation mechanism. The latter suggests that there may be bacterial products and non-living microorganisms in the background of translocation. Plasma, peritoneal fluid and fecal calprotectin levels or the presence of Bactericidal/Permeability-Increasing Protein (PBI) are all associated with complications and poor disease prognosis and may also be used as laboratory markers of BT in cirrhosis. Further studies are needed to assess the applicability of these markers.

Pathological BT is hypothesized to be responsible for the serological response against various carbohydrate and protein components of microbes (appearance of antimicrobial antibodies). Antimicrobial antibodies, like *Saccharomyces cerevisiae* (ASCA) and other glycans (anti-*mannobioside* [AMCA], anti-*laminaribioside* [ALCA] and *anti-chitobioside* [ACCA] antibodies) or anti-OMP antibodies are regarded as characteristic markers for complicated, small bowel Crohn's disease. Patients with untreated celiac disease, however, may also display similar qualitative and quantitative serological response to those with Crohn's disease. As in Crohn's disease, the highest antibody prevalence and titers in celiac patients are associated with the most severe clinical manifestation (malabsorption). This finding is in line with the hypothesis that among celiac patients, malabsorption is the most pronounced clinical consequence of the intestinal damage. Similarly, in cirrhosis inflammation of the small bowels is notable and becomes more pronounced with disease progression. Two-thirds of patients with liver cirrhosis who underwent capsule endoscopy showed mucosal inflammation-like abnormalities. Alterations of small bowel morphology, like partial villous atrophy and mild-to-moderately increased lamina propria infiltration, as well as increased intraepithelial lymphocyte count was also demonstrated in patients with cirrhosis. Fecal calprotectin levels, which indicate intestinal inflammation, and are widely used to evaluate intestinal inflammation in patients with inflammatory bowel disease (IBD) were found increased in liver cirrhosis and concentrations were significantly associated with the severity of inflammation. Therefore, it is reasonable to hypothesize that the antimicrobial antibodies may also be present in patients with cirrhosis and may be associated with the clinical course of the disease. At present, however, there is no data concerning antimicrobial antibodies in liver cirrhosis and its complications.

Regarding serological markers, genetic factors may play a role in the increased susceptibility for pathological BT. There is evidence that functional polymorphisms of PRRs alter the detection and elimination of pathogens, thereby influencing the innate host defense mechanisms. Several clinical studies have shown that single nucleotide polymorphisms (SNP) in the promoter and the encoding regions of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) or toll-like receptor 2 (TLR2) gene increase the risk of developing SBP, however, the results of these studies are not completely consistent. Immune dysfunctions associated with functioning of inherited PRR may increase the risk of both SBP and other systemic bacterial infections in liver cirrhosis. However, neither these membrane-bound and cytoplasmic PRRs associated with non-SBP-type bacterial infections in liver cirrhosis, nor their role in the disease course have been comprehensively analyzed yet.

AIMS

Within the framework of a large, prospective study of patients with cirrhosis, we aim to investigate the serological and genetic factors associated with bacterial translocation (BT) and their role in predicting the development of bacterial infections.

1. We examined the known and clinically significant genetic polymorphisms of certain surface and intracellular pattern recognition receptor proteins (PRRs) of the innate immune system (toll-like receptors [TLR2 and 4] and NOD2/CARD15), with a special emphasize to the following characteristics:
   - their predictive value for the development of cirrhosis-associated bacterial infections and for infection-related mortality
   - their predictive value for disease progression (development of decompensation and/or liver-associated death)
   - their association with the known serological markers of endotoxin exposition

2. We investigated several isotypes of IgA antimicrobial antibodies (ASCA, anti-OMP Plus™ IgA) produced against various surface glycan components and bacterial proteins in patients with liver cirrhosis by
   - determining their frequency and their correlation to the clinical and laboratory severity indicators, and also to the presence of disease-specific complications
   - investigating the differences in the serological response given to other, non-cirrhotic chronic liver diseases
   - analyzing their correlation to known functional PRR polymorphisms in liver cirrhosis and comparing these data with those found in inflammatory bowel diseases
   - assessing their clinical significance in the prediction of bacterial infections and infection-related mortality risk

PATIENTS AND METHODS

Chronic liver diseases

Patients with cirrhosis: Clinical data and biological samples from patients with cirrhosis have been being collected prospectively at Division of Gastroenterology, Department of Internal Medicine at the University of Debrecen since May 1, 2006. A total of 404 patients with cirrhosis were recruited consecutively (male/female: 226/196, median age: 56 years [IQR: 50-64], mean disease duration from diagnosis: 3.9±4.2 years) until December 31, 2010 from the gastroenterology or the hepatology outpatient clinic during regular or unplanned follow-up visits, and also from the inpatient ward when hospitalized with an acute decompensation (AD) episode (non-AD: n=286 and AD: n=118). Prospective follow-up period lasted until December 31, 2013. Within this period several other observational clinical investigations were performed in this patient cohort. Patient numbers and follow-up periods in these clinical studies varied based on the number of patient samples available for laboratory analysis, and also due to the fact whether we included only stable outpatients or also inpatients with AD. According to the internationally accepted definition, acute decompensation was defined if one or more of the following events were present simultaneously: the acute development of ascites (grade II/III according to the criteria of the International Club of Ascites), acute hepatic encephalopathy (i.e., acute neurological deterioration in a patient with previously intact consciousness without a neurological cause), acute gastrointestinal bleeding (in each case diagnosed by upper pan endoscopy and attributed to esophageal varices in accordance with the traditionally used criteria), and/or the presence of systemic bacterial infection. Refractory ascites and chronic hepatic encephalopathy were not considered as an AD episode. Exclusion criteria were: (1) if the patient or their legal representative refused to sign the informed consent form, (2) if the patient was normally treated in another institution and visited us only for a specialist consultation.

The diagnosis of cirrhosis was made based on clinical, biochemical, imaging examinations and, if available, histological findings of liver biopsy. The patients' detailed clinical data were recorded at inclusion. Serum, plasma and blood samples required for the research were collected simultaneously alongside with routine laboratory tests. Clinical data were determined by an in-depth review of patients' medical records using a structured interview. Medical data from before the observational study period were collected retrospectively: age at diagnosis, etiology, presence of esophageal varices, history and date(s) of previous AD episode(s), hepatocellular carcinoma (HCC), extrahepatic comorbidities and cirrhosis-related medication. Disease severity was determined by using liver-oriented scores: Child-Pugh and MELD (Model for End-Stage Liver Disease), and clinical stage of the disease (compensated / decompensated) was also determined. In case of acute decompensation, its type was recorded.

The present PhD dissertation is based on two studies. In *a clinical study with antimicrobial antibodies*, serum samples from 286 stable outpatients were examined and prospective clinical data were analyzed after study completion (April 30, 2009). In *a clinical study with functional genetic polymorphisms of PRRs*, DNA samples from 349 patients, of which 243 were stable outpatients and 106 were patients with AD, were examined and prospective clinical data were extracted for further analyses after the end of the study period (December 31, 2013).

Follow-up patients with cirrhosis: During the follow-up of patients with cirrhosis, the attending gastroenterologist registered the date and type of the AD episode warranting hospital admission and the presence of bacterial infection. The diagnosis of infections in each case was based on the presence of clinical symptoms consistent with the infections, laboratory

parameters (WBC count, CRP, PCT), urine sediment tests and imaging tests (abdominal ultrasound and chest x-ray) and the results of diagnostic sampling (neutrophil granulocyte count determination) when ascites was present. Depending on the test results, microbiological studies were also performed according to the given infection. Hemoculture was also taken in case of sepsis and unidentified source of infection. Among laboratory parameters the following factors supported the diagnosis of bacterial infection: elevated white blood cell count (absolute: >10.8 G/L or relative [in patients with leukopenia]: doubling of previously stable value) with high ratio of neutrophil granulocyte (>76%) and elevated serum CRP (>10.0 mg/L) and/or elevated serum PCT (>0.15 µg/L). Bacterial infections were further described according to conventional criteria. The following bacterial infections were diagnosed:

(1) SBP: ascites, neutrophil granulocyte cell count: >250/mm^3 and/or positive ascites culture in the absence of secondary source of abdominal infection.
(2) Urinary tract infections: symptoms of dysuria, pyuria, (urinary white blood cell count >10/mm^3) and/or positive urine culture findings.
(3) Pneumonia: coughing, positive chest X-ray, positive sputum culture test
(4) Others: skin and soft tissue infections, biliary tract infections, gastroenteritis, osteomyelitis, endocarditis.
(5) Bacterial infections of unknown origin: infectious symptoms, organ-specific focal infection which is not clearly identifiable even with hemoculture-positive bacteremia.

The follow-up period lasted until liver transplantation / death of patient / loss of follow-up (i.e.: if no further patient data were available). In case of non-hepatic mortality, patient data were censored at the time of death. The median follow-up periods were: 425 days [IQR: 107-732] in the clinical study with antimicrobial antibodies, and 1128 days [IQR, 469-1825] in the investigation about functional genetic polymorphisms of PRR. Collected data were transferred and stored in a database. At the end of the study periods, all clinical data was extracted for further analysis.

Control groups

***Patients with autoimmune liver diseases*:** Given the rare incidence of autoimmune liver disease, 266 patients (n=266, male/female 102/164, average age: 51.1 ±16.1 years) were collected from five Hungarian (1st Department of Medicine and 1st Clinic of Pediatrics of Semmelweis University, University of Pécs, Szent Ferenc Hospital, Miskolc and Borsod-Abaúj-Zemplén County Hospital, Miskolc) and one German hepatology center (Otto-von-Guericke University, Magdeburg), and the cases comprised primary biliary cholangitis (PBC, n=153), primary sclerosing cholangitis (PSC, n=59) and autoimmune hepatitis (AIH, n=54). The diagnosis of primary biliary cirrhosis 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 PSC was based on biochemical evidence of cholestasis, characteristic morphological abnormalities of the bile ducts detected by endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP), or if necessary, histology findings. Various causes of secondary sclerotizing cholangitis were excluded in all cases. Using the scoring system of the International AIH Group, the diagnosis of AIH was based on exclusion of other major causes of liver damage including alcoholic, viral, drug- and toxin-induced, as well as hereditary liver diseases.

Patients with other chronic liver diseases (CLD): The non-autoimmune CLD control group consisted of 124 patients with chronic hepatitis C virus infection (chronic HCV, n=124, male/female: 49/75, average age: 53.6 ± 11.7 years). The diagnosis of chronic HCV was based

on ribonucleic acid (RNA) Polymerase Chain Reaction (PCR) test positivity, elevated transaminases (>2xULN for more than 6 months), and - if histological findings were available -, the diagnosis was confirmed by the presence of histological abnormalities.

Inflammatory bowel diseases (IBD): Of patients with Crohn's disease, 513 serum and deoxi-ribonucleic acid (DNA) samples were included (male/female: 211/302, age: 33.0 ± 12.5 years, disease duration: 8.5 ± 7.1 years) from the IBD samples collected by the Hungarian IBD Study Group, to which our gastroenterology study group joined in 2005. The diagnosis of IBD was based on the Lennard-Jones criteria including clinical, endoscopic, radiological, and histopathological examinations. At the time of sampling, the clinical categorization of the disease according to the Montreal Classification was recorded in the database.

Healthy individuals: The healthy control group consisted of 100 healthy blood donors matched by age and gender who did not have any gastrointestinal or liver disease (male/female: 47/53, age: 48.1±15.5 years).

LABORATORY METHODS

Serum, plasma, and whole blood DNA samples were obtained at enrolment and were stored at -70°C until testing.

Molecular genetic examinations: Genetic tests were performed in another institution (by András Bors MD and Prof. Attila Tordai MD, Molecular Diagnostic Laboratory of the National Blood Service).

Gene analysis of NOD2, TLR2 and TLR4: Genomic DNA was extracted from whole-blood samples using the Gentra Puregene Blood Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol. Three alleles of the NOD2 gene variants rs2066844, (p.R702W, NM_022162.2:c2104C>T), rs2066845 (p.G908R; NM_022162.2:c.2722G>C) and rs2066847 (L1007Pfs; NM_022162.2:c.3019dupC) were genotyped using hybridization probes on fluorescence resonance energy transfer (FRET) on a LightCycler 480 (Roche) real-time PCR system, according to Ferreiros-Vidal et al. Gene variant of TLR2 gene rs4696480 (NM_003264.4:c.-148 + 1614T>A) was also genotyped using oligonucleotides according to Oh et al. The gene variant of TLR4 gene rs4986790 (p.D299G; NM_138554.4:c.896A>G) was genotyped using self-designed amplification oligos (TLR4-D299G F: CATCGTTTGGTTCTGGGAG and TLR4-D299G R: TTTACCCTTTCAATAGTCACACTCA), while FRET oligonucleotides were similar to Hamann et al. (TLR4-D299G SENS: CTACTACCTCGATGGTATTATTGACTTATT-6FAM, TLR4-D299G ANCH: Cy5.5-AATTGTTTGACAAATGTTTCTTCATTTTCC- 3′ phosph). Genotyping was technically unsuccessful in two patients for NOD2 analysis, and in one case for TLR2 and TLR4 analysis.

Serological analysis

Study of antimicrobial antibodies: Commercially available enzyme-linked immunosorbent assay kits (ELISA) (QUANTA Lite™ INOVA Diagnostics, San Diego, CA and Hycult Biotechnology, Uden, Netherlands) were used to detect the presence of ASCA IgA and IgG, anti-OMP Plus™ IgA and endotoxin core IgA antibodies (EndoCab). In the case of ASCA, the antibodies of the two different isotypes were evaluated separately. The results were presented in arbitrary units for each antimicrobial antibody using the equation provided by the manufacturer (cut-off value for positivity was 25U for ASCA and anti-OMP Plus™, and 195 U/ml for EndoCab), and were documented both in absolute values and as a percentage of the positive cases.

ASCA is an antibody directing against the mannose antigen in the outer membrane of *Saccharomyces cerevisiae*, while in the case of anti-OMP Plus™, the antigen is a mixture of proteins from Gram-negative (GNB) and Gram-positive bacteria (exact formulation encrypted by the manufacturer, INOVA Diagnostics, San Diego, CA, US), but differs from porin protein C of *Escherichia coli*. Therefore, anti-OMP Plus™ and anti-OmpC antibodies (Prometheus Laboratories Inc., San Diego, CA, US) are also diverse. EndoCab directs against a mixture of incomplete endotoxins of four different species - *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli* and *Klebsiella aerogenes*.

In order to evaluate the variation in the antimicrobial antibody levels, duplicate serum samples were taken from a subgroup of patients with cirrhosis (n=62) at different time point with the median time interval of 204 days [IQR, 71-245]. Determination of the acute phase protein (APP): lipopolysaccharide binding protein [LBP] was determined from sera with the commercially available ELISA method (Hycult Biotechnology, Uden, Netherlands). The lower sensitivity cut-off was 0,1 µg/L.

STATISTICAL METHODS

For statistical analysis, Prism 5 (GraphPad Software Inc., San Diego CA, USA) and SPSS 13.0 (SPSS Inc, Chicago, IL, USA) were used, with the help of a statistician (Dr. Elek Dinya). Variables were tested for normality with Shapiro-Wilk's W test. In functional polymorphism examinations, genotypes were tested for deviations from Hardy-Weinberg equilibrium test. Categorical variables were compared with Fisher's exact test and χ^2-test with Yates correction as appropriate. Results are expressed as odds ratio (OR) with 95% confidence intervals (95% CI). In the case of APP, autoantibodies and antimicrobial serological markers, continuous variables were compared with D-test and ANOVA with post hoc Scheffe; and Pearson's or Spearman's rank order correlation was calculated. When necessary, prevalence was determined and used in the calculations. Logistic regression analysis was used to conduct multivariate analysis of binary factors, results were expressed as *probability value* and OR. The correlation between the individual factors and the course of disease was assessed using Kaplan-Meier analysis, while the significance of the subgroups was determined by LogRank and Breslow tests. Multivariate analysis was performed with Cox-regression analysis. In the univariate analysis, $p<0.1$ factors and some previously selected factors were included in the multivariate analysis. Unless otherwise specified, $p<0.05$ was considered as significant. Raw data were presented as "mean±standard deviation", "median (quartile)" and "case number (%)".

ETHICAL CONSIDERATIONS

The study protocols were approved by the Regional and Institutional Research Committee of University of Debrecen and by the National Scientific and Research Ethics Committee. Ethical license numbers: DEOEC RKEB/IKEB 2006-2007, 2595-2007, 2735-2008, 2860-2008, 3515-2011, 3514-2011; ETT-TUKEB 321-81/2005-1018-EKU, 80/2007-EKU, 25403/2011-EKU, 26186/2011-EKU. Each patient or legal representative was informed of the nature of the study and signed an informed consent form.

RESULTS

1. Investigation on functional polymorphisms of membrane-bound and intracellular pattern recognition receptor proteins (PRRs) in liver cirrhosis.

Genotype distribution of various functional polymorphisms of PRRs in cirrhosis

The prevalence of NOD2 (R702W, G908R and L1007PfsinsC), TLR2 (−16934T>A) and TLR4 (D299G) genotypes in cirrhosis was distributed as follows: NOD2 variant genotype is 16.4%, while wild type is 83.6%; TLR2 TT: 24.8%, TA: 44.4% and AA genotype: 30.8%; TLR4 AA 93.1%, AG 6.6% and GG 0.3%. The incidence rates of each genotypes were not different between stable outpatients with cirrhosis and hospitalized patients with acute decompensation.

Further analysis of clinical and laboratory characteristic of outpatients revealed that age, gender, comorbidities, and the presence of HCC, etiology or severity of cirrhosis were not different across the various PRR genotype subgroups. Co-medications at enrolment comprising the use of proton pump inhibitors (PPI), non-selective beta blocker (NSSB), and secondary antibiotic prophylaxis with either norfloxacin for prevention of SBP or rifaximin for prevention of HE were also not different among patients with the genetic variant of NOD2, TLR2, and TLR4 and with the wild type.

Non-spontaneous bacterial peritonitis (SBP) type bacterial infections and their risk factors

During the follow-up period, eighty-five (35%) of the included cirrhotic patients encountered a non-SBP type bacterial infection episode. The median time to the development of a first infection was 581 [207-803] days. Urinary tract infection was the most commonly diagnosed infection and accounted for 43.5% (n=37) of the events. Other sites of BI were as follows: pneumonia (18.8%), erysipelas (10.6%), acute bronchitis (5.9%), cholangitis (3.5%), bacteremia (3,5%), gastroenteritis (1.2%). In nine cases (10.6%), the localization of BI could not be determined. 2.4 % of the cases were multifocal. Microbiological analysis was performed in 35 (41.2%) cases. Based on culture results (n=17), bacteria were Gram-negative in 76.5% and Gram-positive in 23.5%. The distributions of the isolated pathogens were as follows: *Escherichia coli (n=6), Enterococcus faecalis (n=3), Klebsiella pneumoniae (n=5), Pseudomonas aeruginosa (n=1),* and *Citrobacter freundii (n=1)*. No fungal infection occurred.

Functional polymorphisms of PRR gene as the risk factors of non-SBP type bacterial infections

Patients with any risk variants of NOD2, TLR2 or TLR4 genes did not have an increased cumulative risk for a non-SBP type BI episode during follow-up (pLogRank=0.107, pLogRank=0.439 and pLogRank=0.535, respectively); not even when stratified according to severity (compensated vs. decompensated cirrhosis). Decompensated disease was due to the presence of ascites. There was no difference between patients with both TLR2 and at least one NOD2 risk variant (n=10, combined risk factor) compared to patients with only one risk variant (pLogRank=0,397). There was no rationale for testing the potential effect of the TLR4 and NOD2 variant combination because only one patient carried both variant genotypes.

The presence or absence of a NOD2, TLR2 or TLR4 variant did not affect the type of pathogens causing BI, either Gram-negative or Gram-positive), or the location of BI.

NOD2, TLR2 and TLR4 genotypes showed no association with the risk of infection-related 30-day and 90-day mortality ($OR_{30\text{-day}}$ [95%CI]: 0.14 [0.02-1.11, p=0.061] and $OR_{90\text{-day}}$ [95%CI]: 0.24 [0.05-1.14, p=0.073] in terms of NOD2 variant; while $OR_{30\text{-day}}$ [95%CI]: 0.67 [0.2-2.22, p=0.509] and $OR_{90\text{-day}}$ [95%CI]: 0.43 [0.13-1.37, p=0.153] TLR2 (TA) and $OR_{30\text{-day}}$ [95%CI]: 0.85 [0.27-2.63, p=0.773] and $OR_{90\text{-day}}$ [95%CI]: 0.64 [0.22-1.88, p=0.414] as TLR2 (TT) for reference; $OR_{30\text{-day}}$ [95%CI]: 4.07 [0.83-19.84, p=0.082] and $OR_{90\text{-day}}$ [95%CI]: 3.13 [0.65-15,11, p=0.155] for the TLR4 (AG) genotype.

Clinical risk factors in the development of non-SBP type bacterial infections in cirrhosis
Non-SBP-type BI developed in 49.6±4.1% of the patients (cumulative probability [CP] and standard error [SE]). Of the clinical factors, comorbidity (57.8±5,8%, pLogRank<0.038), PPI use at enrolment (60.5±5.9%, *pLogRank<0.009*) were all associated with an increased cumulative probability of non-SPB type BI episodes during the follow-up period. Of the patients with a prior history of BI, 66.5±6.3% developed another BI episode, as compared to 39.7±5.1% of those with no such history (*pLogRank<0.001*). Regarding advanced disease stage, similar results were found if advanced disease stage was depicted either by the presence of ascites (65.2±6.6% vs. 42.0±5.1%, *pLogRank<0.001*), Child-Pugh stage B/C (68±6.1% vs. 38.1±5.2%, *pLogRank<0.001*), or by decompensated clinical stage (58.8±5.7% vs. 40.8±5.8, *pLogRank=0.01*).

The combination of these two relevant clinical factors, the prior history of BI and advanced disease stage revealed important findings. On the one hand, prior history of a BI episode significantly increased the probability of any subsequent development of another BI event, regardless of disease severity. On the other hand, prior history of BI in patients with compensated cirrhosis was associated with the same cumulative probability of BI occurrence, as in decompensated stage without prior history of BI (57.3±8.7% and 51.0±9.9%). An even higher cumulative probability of BI (80.3±7.7%, HR: 5.57, 95%CI: 3.23-9.59, *p<0.001*) was resulted by the combined presence of both clinical risk factors.

Multivariate analysis
Taking all significant clinical factors of the univariate analysis into account, multivariate Cox-regression analysis indicated that presence of ascites (HR [95% CI]: 1.71 [1.08- 2.7], increased MELD score (1.08 [1.02-1.15]) and any prior BI episode (2.02 [1.3-3.14]) were independently associated with the risk of non-SBP type BI development during follow-up.

Spontaneous bacterial peritonitis (SBP) type bacterial infections and their risk factors
During the follow-up period, 22.7% (20/88) of the patients with ascites developed community acquired SBP. Of the where microbiological samples were taken, 36.4% (4/11) was culture positive SBP, while 63.6% (7/11) was culture negative. The identified pathogens were as follows: *Klebsiella pneumonia (n=1), Enterococcus faecalis (n=1)* and *Citrobacter freundii (n=2).* In terms of Gram staining, 75% of bacteria was GNB while 25% was found GPB. The median time to the development of SBP was 340 (126-662) days. Among the tested PRR variants, the presence of NOD2 risk allele variants was associated with an increased cumulative probability of SBP (76.9±19.9% vs. 30.9±6.9%, *pLogRank=0.047*). There was no significant difference in the MELD scores of patients with and without any NOD2 risk allele variants (median [IQR]: 14 [9-16] vs. 13 [10-15], *p=0.874*). Prior SBP episode was also a risk factor for SBP development (*pLogRank=0.048*). However, there was no difference in the cumulative probability of developing SBP in terms of TLR2 and TLR4 variants.

Association of functional polymorphisms of PRR genes with serological markers of bacterial translocation (BT) According to the examined PRR genotypes, serum level of LBP and frequencies of IgA type antibodies (EndoCab) directed against various gut microbial components were not different.

Predictive value of functional PRR gene polymorphisms in the development of decompensated disease stage
Of the patients with a compensated clinical stage at enrolment 31.4% (38/121) developed any type of decompensation event (ascites, esophageal varices, or hepatic encephalopathy). The median time to the development of a first decompensation was 540 (IQR, 140-913) days. Neither NOD2 risk variants (*pLogRank=0.681*), nor TLR2 and TLR4 polymorphisms (*pLogRank=0.068* and *0.249*) were risk factors of clinical decompensation of cirrhosis.

Predictive value of functional PRR gene polymorphisms in total mortality associated with cirrhosis

Total liver-related mortality was 33.7% (82/243) in the overall patient cohort. Survival was significantly worse in patients with advanced liver disease (Child-Pugh B/C), presence of ascites (*pLogRank*< 0.001 for both) and prior BI episode (pLogRank=0.033). Neither NOD2 variant genotypes (*pLogRank*=0.785), nor TLR2 and TLR4 polymorphisms (pLogRank=0.682 and 0.732) were associated with overall survival.

2. Investigation of antimicrobial antibodies in liver cirrhosis

Prevalence rates of Anti-Saccharomyces cerevisiae (ASCA) and anti-OMP Plus™ antibodies in patients with cirrhosis

Prevalence of ASCA IgA and IgG, as well as anti-OMP Plus™ IgA was examined in liver cirrhosis and other non-cirrhotic CLDs.

ASCA IgA/IgG occurred in 38.5% in liver cirrhosis, while this rate was 62.6% for anti-OMP Plus™ IgA. The rate of ASCA IgA/G and anti-OMP Plus™ IgA seropositivity was greatly elevated in patients with liver cirrhosis compared to healthy controls ($OR_{ASCAeither}$: 3.28, 95%CI: 1.83-5.89; $OR_{anti\text{-}OMP\ PlusTM}$: 6.69, 95%CI: 3.88-11.55) or to CLDs without cirrhosis (*p<0.001* for both). The rates of ASCA and anti-OMP Plus™ positivity were not different overall in patients with autoimmune liver disease without cirrhosis, as compared to the controls. However, evaluating PBC, PSC and AIH patient groups separately, we found that the frequency of ASCA IgA and/or IgG positivity was also higher in the group of patients with PSC (p=0.04) - but not in those with primary biliary cirrhosis or AIH - than in the healthy control group. Within the autoimmune cohort, no similar tendency was observed for anti-OMP Plus™ antibodies. In patients with cirrhosis, the serological profile was not different based on alcoholic and non-alcoholic etiology. Of patients with cirrhosis, 10.4% were triple positive for ASCA IgA, ASCA IgG and anti-OMP Plus™ IgA antibodies, as compared with only 1% of the control subjects *(p<0.0001)*. Only one patient was triple-positive in the AIH group and none in the chronic HCV group.

Association of antimicrobial antibodies with disease severity and disease-specific complications

Seropositivity rates of both ASCA IgA and anti-OMP Plus™ IgA increased gradually according to disease severity, represented by the Child-Pugh stage (p<0.001). 59.2% of patients with Child C cirrhosis were positive for ASCA IgA/IgG while anti-OMP Plus™ IgA occurred in 84.2% of this patient group. Furthermore, 18.4% of patients were triple positive for the examined microbial antibodies, compared with only 4.2% in Child A and 10.3% in Child B groups (*p<0.01* for both). The same correlation was found if the severity was calculated for the MELD inter-quartile (IQR), (1st quartile: 1.7%, 2nd quartile: 6.6%, 3rd quartile: 13.3% and 4th quartile: 20.3%, *p<0.01*).

Similar to the rates of seropositivity, the more severe the disease, the higher were the titers of the ASCA IgA and anti-OMP Plus™ IgA, but not the titer of ASCA IgG. The presence of ASCA and anti-OMP Plus™ antibodies as well as multiple seropositivity were associated with the presence of ascites ($OR_{ASCAeither}$: 1.93; 95%CI: 1.19-3.14 and $OR_{anti\text{-}OMP}$: 3.08; 95%CI: 1.86-5.11, p<0.001 for both). Apart from seropositivity, significantly higher ASCA IgA and anti-OMP Plus™ IgA titer values were observed in patients with ascites (*p<0.001*, for both) than those without. ASCA IgG titers did not differ in the presence of ascites.

Median serum LBP levels were not statistically different between patients with liver cirrhosis and healthy subjects (21.860 vs. 19.333 ng/mL, p=0.08). No correlation was found between LBP levels and the disease severity or the presence of ascites. Furthermore, serum PBP levels were not different in the presence of ASCA IgA/IgG or anti-OMP Plus™ antibodies.

Correlation between antimicrobial antibody formation and NOD2 risk variants

In cirrhosis, nether ASCA isotypes, nor anti-OMP Plus™ antibody formations were associated with the presence of NOD2 risk variants. This was a significant difference compared to Crohn's disease. Like cirrhosis, Crohn's disease is also associated with increased chronic BT, as well as typically increased antimicrobial antibody production. In Crohn's disease, both ASCA IgA (58.6% vs. 35.8%, *p=0.002*) and ASCA IgG (64.5% vs. 41.7%, *p<0.001*) were more common in patients with NOD2 risk allele than in those with the wild type. Similar tendency was observed for anti-OMP Plus™ (35.5% vs. 24.6%, *p=0.061*).

Predicting value of antimicrobial antibodies in the development of cirrhosis-related bacterial infections

In the presence of ASCA IgA and anti-OMP Plus™ antibodies, the cumulative risk of bacterial infections was higher than in their absence (ASCA IgA: 54.6±6.3% vs. 29.1±3.9%, *p<0.001* for both; and anti-OMP Plus™ IgA: 42.9±4.5% vs. 28.2±5.2%, *p=0.033*). However, no difference was observed in the cumulative probability of development of bacterial infections in the presence or absence of ASCA IgG antibody (38.5±3.5% vs. 34.4±5.0%, p=0.466). Among patients with compensated cirrhosis (absence of ascites), in ASCA IgA antibody positive cases the cumulative probability of the development of further infections was the same as in patients without ASCA IgA in the decompensated stage (presence of ascites) (42.7±9.1% and 43.1± 6.9%). In the presence of both ascites and serological antigens, the cumulative risk of infections was even higher (68.5±6.0%, HR: 6.03, 95%CI: 3.11-11.68, p<0.001).

Elevated serum total IgA levels were found to be similar to IgA isotype antimicrobial antibodies (70.7±10.1% vs. 63.4±6.0%; HR: 1.75, 95%CI: 1.06-2.9, p=0.030).

After analyzing the correlation between the IgA-specific isotypes of antimicrobial antibodies and total IgA levels, and the onset of bacterial infections using multivariate Cox regression model, we found that besides disease severity – which was defined either by the Child-Pugh score (HRadj.: 3.00, p<0.001 for Child C vs. A) or the presence of ascites (HRadj.: 2.4, p<0.001), or the increasing MELD score (HRadj.: 1.08, p<0.001 for an increase of 1 in the MELD score) – only the ASCA IgA antibodies proved to be independent risk factors from all the specific antibodies (HRadj. 1.92-2.18, p<0.01, adjusted for the three different disease severity variables), while the anti-OMP Plus™ IgA or the elevated total IgA level did not.

DISCUSSION

In cirrhosis of the liver, apart from the SBP (a characteristic type of infection in patients with ascites), other non-SBP type bacterial infections have significant prognostic implications. Therefore, individual risk stratification for BI is an important clinical issue. If more accurate identification of high-risk patients were possible, morbidity and mortality could be reduced by introducing antibiotic or non-antibiotic preventive treatment strategies, and/or establishing a closer follow-up protocol. Former clinical studies of functional PRR gene variants in cirrhosis predominantly focused on the development of SBP in patients with ascites. In contrast, in the present study we comprehensively assessed the predictive value of various functional SNPs of three different PRR genes simultaneously in a large prospective cohort, comprising the whole severity spectrum of cirrhosis, with a special emphasis on the development of non-SBP type BIs.

In our patient cohort, the frequencies of PRR gene variants were comparable with other cirrhotic patient cohorts, and with healthy Caucasians.

Similar to most of the previous studies, the presence of NOD2 allele variants was a risk factor for SBP in our patient population as well. A recent comprehensive study in patients with decompensated cirrhosis, however, did not demonstrate the role of NOD2 variant in mediating susceptibility for SBP. In our present study, development of SBP did not differ in patients with TLR2 (-16934 T>A, rs4696480) and TLR4 (D299G, rs4986790) polymorphisms. This latter finding is a novelty and has not been previously investigated in the literature. The association of SBP with various TLR2 genotypes is controversial in the published literature. In the studies of *Nischalke et al*. and *Lutz et al.*, TLR2 (-16934 TT) genotype, but not TLR2 R753Q and P631H mutations, were associated with SBP. In contrast, *Bruns et al.* reported that not the TLR2 (-16934T>A), but the TLR2 R753Q polymorphism increased the risk of SBP. A limitation of our study compared to previous cohorts was the relatively low number of patients with ascites (n=88). The present study did not allow for an in-depth analysis of the association of PRR variants with different types of SBP (culture-negative, culture-positive or bacterascites). There were twenty cases of SBP during the follow-up period, and only half of them was cultured.

The PRR gene variants examined in our study have been showed in non-cirrhotic patient cohort to have a special role in susceptibility to bacterial infections and sepsis in patients with acquired immune deficiency (namely, acute leukemia or allogeneic stem cell transplantation). Moreover, in critically ill patients, the NOD2/TLR4 combination was associated with higher rate of bacteremia. A single retrospective study (n=111) reported that in advanced liver disease, the TLR4 (D299G, rs4986790) variant was identified to increase overall BI rates. At the same time, another TLR4 variant (c.+1196C/T, rs4986791) did not increase the risk of BI in a large retrospective cohort (n=336), including a validation cohort with same sample size. In our investigation, none of the examined PRR variants were associated with higher risk of non-SBP type bacterial infections.

The two main members of the PRR family – TLRs and NOD like receptors – act synergistically in the initiation of host innate immune response to BI. Nevertheless, neither of the NOD2, TLR2 and TLR4 gene variant combinations showed increased BI susceptibility in our cohort. The limitation of the present study is that the size of the examined cohort did not allow for an analysis of the association of bacterial infections and TLR4 polymorphisms. The detection of possible associations between TLR4 and non-SBP type bacterial infections would require further investigation in a larger patient cohort than ours. In our study, we distinguished seven different localized infections; however, the limited number of incident cases in the different subgroups did not make possible a more subtle assessment of the potential role of PRR variants in the development of certain types of infection.

The strength of our study is that the whole disease severity spectrum of cirrhosis was represented, allowing for an in-depth analysis of the interaction of PRR gene variants and BI development in various disease severity groups. Cirrhosis associated immuno-deficiency syndrome (CAID) is a dynamic process evolving with the natural history of progression to end stage liver disease. Thus, the impact of an inherited risk for a BI might be different in early vs advanced cirrhosis, owing to limited compensatory mechanism. However, in our patient cohort, PRR gene variants were not associated with a higher risk of non-SBP type BI in any subgroups of various disease severity. These results confirm that acquired immune deficiency state in cirrhosis is more significantly a risk factor than the presence of functional genetic polymorphisms in the development of BI.

The most notable finding of our study was that a prior episode of BI was a risk factor for the development of a subsequent BI episode. This suggests the presence of other persistent host factors that modulate an individual's susceptibility for bacterial infections. Interestingly, this association was observed both in early and in advanced stages. History of a prior BI episode and an advances disease stage had similar impact on the infectious risk. Pathological BT is strongly associated with several clinically relevant complications in cirrhosis. There is evidence that variants of the NOD2 gene and various TLR polymorphisms contribute to BT in patients with Crohn's disease. Likewise, in patients with decompensated cirrhosis, an increased translocation of bacterial DNA fragments into ascites fluid was found in the presence of the NOD2 risk variant p.G908R. Moreover, there was increased transition of pathological BT to culture positive SBP in the case of the same NOD2 variant. TLR2 (-16934 T>A, rs4696480) and TLR4 (D299G, rs4986790) polymorphisms were associated with an increased systemic antigen burden as well, described by the serum level of lipoteichoic acid, LPS, and bacterial DNA.

In our study we applied both serological and clinical approaches to assess the impact of PRR genetic variants to BT. First, we examined the effect of NOD and TLR SNPs on the serological response to BT; however, we used different serological markers than in the study of *Piñero et al.* The frequency of IgA type antimicrobial antibodies and LBP levels in our study did not differ between various PRR genotypes; neither in the entire cohort, nor in the subgroups of patients with or without ascites. Second, we hypothesized that if PRR genetic variants were linked to BT, they would be associated with enhanced disease progression, like early onset of decompensation or increased liver-related mortality. The different polymorphisms of NOD2 and TLR2 and TLR4 genes did not affect these advert events. Our study is the first to consider the effect of PRR gene variants on the development of a decompensation event in cirrhosis.

The effect of PRR gene variants on mortality has been analyzed previously but yielded conflicting results. *Appenrodt et al.* found a fourfold increased risk in cirrhotic patients with NOD2 risk alleles. Consistent with our results, *Bruns et al.* did not report an increased hazard of death related to the same variants of NOD2.

To our knowledge, serological markers such as antimicrobial antibodies recognized by PRR, predicting the development of BT, may also predict bacterial infections in cirrhosis. This is the first report to investigate the complex association between complicated small bowel Crohn's disease and antimicrobial serological responses in a large cohort of patients suffering from various chronic liver diseases, including cirrhotic and non-cirrhotic patient groups. We confirmed that in chronic liver diseases associated with cirrhosis, the formation of antimicrobial antibodies, such as ASCA and anti-OMP Plus^{TM}, is common and unrelated to the etiology. However, in the absence of liver cirrhosis in CLD, increased antibody production was not observed compared to the healthy control group. The rate of ASCA positivity in patients with alcoholic liver disease and chronic HCV was equal to the findings of previous studies. In the background of antimicrobial antibody formation - especially the ASCA -, the role of genetic susceptibility is considered important in Crohn's disease. ASCA formation is associated with the presence of certain PRR polymorphisms, such as NOD2. We managed to confirm this

association in our large patient cohort with Crohn's disease. Inadequate recognition of the components of the gut flora results in sustained absorption of bacterial antigens which may provoke and maintain increased antibody formation regardless of the disease severity and mucosal inflammation. However, it is worth mentioning that ASCA prevalence was also high in the patient cohort, carrying the wild type of NOD2/CARD15 allele according to both our own and other reported studies. This suggests that genetically based loss of tolerance towards the gut flora is not the only mechanism triggering the antimicrobial antibody production. In our cirrhotic patient cohort, neither ASCA nor anti-OMP Plus™ antibody formation were associated with PRR genotypes. Another supporting finding is that the prevalence of ASCA is also high in untreated coeliac disease without higher percentage of variant NOD2/CARD15 alleles as compared to the control group. We hypothesized that acquired BT plays a central role in the formation of antimicrobial antibodies in cirrhosis, i.e., the sustained bacterial translocation from the gut to the systemic circulation as a consequence of the enhanced intestinal permeability and compromised mucosal immunity. This hypothesis is supported by the fact that in our study, the increase of both the seropositivity rates of antimicrobial antibodies and their titers were associated with the progression of cirrhosis according to Child-Pugh stages and the presence of portal hypertension. Gram-negative bacteria are the most frequently translocating bacteria in advanced liver disease or found in the ascites. Anti-OMP Plus™ IgA - which includes fractions targeting bacterial proteins derived from Gram negative gut bacteria - were detected in 62.6% of the cirrhotic cohort. *Mehta et al.* demonstrated that another antibody (anti-Gal) directed to the surface antigen of Gram-negative bacteria are significantly associated with advanced fibrosis (≥ stage III) regardless of the etiology of CLD. Patients with increased level of anti-Gal antibody had increased levels of endotoxin and other markers of bacterial exposure. Based on our findings, PSC was the only subgroup within CLD in which we detected significantly higher antimicrobial prevalence (35%). The pathogenetic mechanisms of PSC also support our hypothesis that antimicrobial antibody formation is a consequence of sustained bacterial translocation. Because of the development of biliary stenotic lesions in PSC, the likelihood of infection is relatively high. The frequency of cholangitis was significantly correlated with the presence of stenotic lesions.

Previously, LBP was suggested as a serological marker of BT; however, in the present study this theory could not be confirmed. Serum LPB levels did not differ between stable outpatients with cirrhosis and healthy control subjects. Furthermore, our study group reported that in cirrhosis LBP - similar to CRP and PCT - should be used for detecting bacterial infections, although its diagnostic efficacy falls behind that of the other two APPs. No association was found between LBP levels and antimicrobial antibody seropositivity.

Among the antimicrobial antibodies, the presence of IgA isotypes predicted the development of systemic bacterial infections associated with cirrhosis in our follow-up clinical trial. In the presence of ASCA IgA, the development of infections was significantly frequent, and the same tendency was observed for anti-OMP Plus™ IgA, although, in the latter case, the difference was not considered significant. These data may provide further evidence on the fact that due to local immunodeficiency, translocation of bacteria and/or antigens to the systemic circulation plays a principal role in the enhanced development of mainly IgA-type antimicrobial antibody formation. The IgA secretion of the B-cells in gut-associated lymphoid tissue (GALT) happens in the presence of microbial antigens. Dendritic cells continuously present intestinal bacteria to the immune system. IgA class switching ('switch recombination') occurs via both T-cell-dependent and T-cell independent pathways. In addition, conserved molecular structures of bacteria, such as CpG oligonucleotides, are able to directly activate IgA production of B-cells with mediation of TLR9. The activated B-cells, after undergoing a shift in their IgA isotype, migrate to the mesenterial lymph nodes through the visceral lymphatics, and eventually reach the systemic circulation through the thoracic duct. Later they return to mucosal surfaces using special 'homing' receptors. Since these activated, mucosal B-cells

reach the systemic circulation through the thoracic duct, an increased local IgA production due to bacterial translocation can be detected also in the peripheral circulation.

Based on our results, the serological response to the cellular glycan components and proteins of intestinal bacteria can be concluded to be specific only for the course of the BT. Our study group has newly identified IgA isotype antimicrobial antibodies as the serological markers of BT. Further analysis of the association between ASCA IgA and the development of infections proves that these antibodies are independent of disease severity and the presence of comorbidities, therefore, they can be applied as serological risk factors of the disease.

We hypothesize that ASCA is unlikely to have pathogenetic role in the disease progression (injury, fibrogenesis) and the bacterial complications, however, their increased formation indicates an underlying pathogenetic course – the BT itself -, which ultimately will lead to the progression of disease. The presence of IgA isotype antimicrobial antibodies is not only the consequence, but also the cause of chronic inflammation. A newly identified population of B-cells (immunosuppressive IgA+ B-cells) are reported in the literature. The progression of liver cirrhosis is characterized by the dynamic coexistence of systemic inflammatory response and immunodeficiency which known as CAID syndrome. As liver disease worsens, BT leads to proinflammatory immune responses, however, due to continuous and increasing bacterial exposure, the immune system is depleted and the immunodeficient CAID phenotype takes over which is associated with the increase of production of anti-inflammatory cytokines (such as IL-10) and of the expression of negative regulators (such as PD-L1). B-cell population with regulatory properties may be one the key factors in these processes. Accumulation of IgA-producing B-cells in non-alcoholic steatohepatitis (NASH) was reported in studies with human and mouse models. These cells also express PD-L1 and IL-10 directly inhibiting the function of anti-tumor cytotoxic T-cells and promoting the development of hepatocellular carcinoma (HCC). By eliminating or inhibiting the immunosuppressive IgA+ B-cells – which form a separate population from the well-known intestinal B-cells maintaining microbial homeostasis – produced in chronic liver diseases, the risk of cancer formation can be reduced. Another study showed an increase in PD-L1+ expression of B-cells in the peripheral blood of patients with sepsis. According to the reported data, the increase in the number of immunosuppressive B-cells in the intestinal tract, the liver tissue and the systemic circulation is hypothesized to be detected also in patients with alcoholic cirrhosis. Their presence may be associated with a higher incidence of systemic infections. The latter may be supported by the fact that in the course of sepsis, activation of the immune system is followed by immunosuppression like in CAID. In the future, by eliminating or inhibiting (PD-L1) the immunosuppressive IgA+ B-cells the risk of systemic infections can be decreased, and the proper function of the immune system restored. Based on our data, investigating the presence of immunosuppressive IgA+ B-cells in the systemic circulation and also various tissue compartments (e.g. GGI tract, liver), and their comparison with production of antimicrobial antibodies seems an import task.

NOVEL SCIENTIFIC FINDINGS

By investigating the clinical factors of bacterial infections in liver cirrhosis we could confirm that history of a previous infectious episode is a novel risk factor, it is independent of disease severity and is equally significant to it. Regarding their effects, these clinical risk factors proved to be additive.

The functional polymorphisms of various cellular pattern recognition receptor (PRR) genes which represent the innate susceptibility to the development of bacterial infections in liver cirrhosis are less important than the acquired susceptibility factors leading to cirrhosis-associated immunodeficiency syndrome (CAID), both in early and in advanced disease stages.

The well-known *NOD2* risk variants [L1007fsinsC −/C, R702W C>T or G908R G>C] and *TLR2* [−16934T>A] or *TLR4* [D299G] did not contribute significantly to the course of pathologic bacterial translocation (BT) in liver cirrhosis. This theory is supported by the lack of correlation between the PRR gene polymorphisms and either the clinical manifestation of BT, the development of decompensated cirrhosis, or the liver-related mortality.

By simultaneously investigating the serological response targeted against the surface carbohydrate and protein components of gut bacteria in both inflammatory bowel diseases (IBD) and liver cirrhosis, we could confirm that this response is specific not to the disease, but to the process of BT itself. The IgA isotype antimicrobial antibodies (the anti-*Saccharomyces cerevisiae* (ASCA) and the anti-OMP Plus™) are thus novel serological markers of pathologic BT, first identified by us.

The frequency of IgA isotype antimicrobial antibodies in liver cirrhosis is high and is correlated to the advanced disease stage and presence of portal hypertension. In chronic liver diseases without liver cirrhosis – except for primary sclerotizing cholangitis - the rate of antibody production was not significantly different than in the healthy controls.

The presence of IgA isotype ASCA antibodies in liver cirrhosis was identified as a novel serological risk factor of bacterial infections in a prospective clinical study.

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List of publications related to the dissertation

1. **Dinya, T.**, Tornai, T. I., Vitális, Z., Tornai, I., Balogh, B., Tornai, D., Antal-Szalmás, P., Sümegi, A., Andrikovics, H., Bors, A., Tordai, A., Papp, M.: Functional polymorphisms of innate immunity receptors are not risk factors for the non-SBP type bacterial infections in cirrhosis.
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