SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

SIGNIFICANCE OF HAPTOGLOBIN POLYMOPHISM AND MANNOSE-BINDING LECTIN DEFICIENCY IN PREDICTION OF BACTERIAL INFECTIONS IN LIVER CIRRHOSIS

by

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The Examination takes place in the Library of Department of Ophthalmology, Medical and Health Science Center, University of Debrecen, 05. September 2011. at 11.00.

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen 05. September, 2011. at 13.00.

Introduction

1.1 Liver cirrhosis and infection

Treatment of liver cirrhosis is a great medical problem all over the world. In Hungary the most important etiological factor is alcohol consumption. According to observations of the last two decades the importance of bacterial infections in treatment of this disease became obvious. Liver cirrhosis is the most common immune deficient state, patients are susceptible for infections that may progrediate into severe forms. Infections impair liver function, trigger complications of liver cirrhosis, including variceal bleeding, hepatic encephalopathy, renal failure, and impairment in clotting factors, and thus become important factors of mortality. Bacterial infections have been observed in 32-34% of hospitalized cirrhotic patients. Advanced stage of liver cirrhosis (Child–Pugh C) and gastrointestinal bleeding enhance the risk of infections, while on the other hand there are convincing data that during an infection the risk of gastrointestinal bleeding is much higher and it's often associated with the failure to control bleeding, with early rebleeding and increased mortality rate. The mortality connected to infection is more than twice higher than mortality of cirrhotic patients without infection.
About half of infections have atypical clinical appearance or no signs. The diagnosis is often established too late. Adequate therapy and prophylaxis have great importance in treatment of liver cirrhosis. It is proven that long term prophylactic administration of antibiotics (2x200 mg norfloxacin) reduces the incidence of infection and improves survival rate, however, its application is limited by the possibility of the development of antibiotic resistance. It would be very important to identify the most susceptible population that surly profits from antibiotic prophylaxis and to avoid any unnecessary antibacterial treatment Advanced disease, reflected by Child-Pugh stage, and the presence of gastrointestinal hemorrhage are the only sustainable and reproducible risk factors for bacterial infections, that increase the risk of mortality. Knowledge of other risk factors may allow for preventive interventions targeting at minimizing the infection-related mortality.

Compromised host defense, prolonged bacteremia

The liver is a bacterial filter and Kupffer cells play an important role in eliminating intestinal bacteria that can cross the bowel wall. In liver cirrhosis there is a marked depression of reticuloendothelial systemic (RES) function, the number and function of Kupffer cells are

decreased. Additionally, the collateral circulation by-passes a certain part of the blood-volume getting directly into the systemic circulation.

Multiple levels of immune dysfunction have been identified in cirrhotic patients parallel with the impairment of hepatic capacity. The deficient opsonin and complement system reduce the phagocytic activity. Prolonged endotoxin stimulation leads to immune paralysis in monocytes. There are data that show the defect of adaptive immunity, too (reduced immunglobulin A contain of stool, T cell depletion). As a consequence of impaired host defense, during local infections or more often simply by intestinal translocation, bacteria get into the circulation causing frequent and long term bacteremia. Colonization in different localization of the body leads to spontaneous bacterial peritonitis (SBP) meningitis, endocarditis, sepsis having especially high mortality rate.

Intestinal bacterial translocation

Bacterial translocation (BT) is a normal physiologic event that means the passage of not only viable bacteria but also endotoxins, antigens and bacterial DNA from the intestinal lumen to the circulation. In pathological states they can induce systemic immune response. Increased portal pressure, altered microflora (bacterial overgrowth), impaired intestinal motility and host defense causing pathological translocation lead to increased bacterial invasion to the circulation. In liver cirrhosis the most important infection port is the injured gut mucosa being the source of bacteremia mainly caused by enteric flora, most often Gram-negative bacteria (GNB) and source of high endotoxin level in the sera that have great importance in maintaining portal hypertension. BT induces mainly spontaneous bacterial peritonitis, but it is a possible causal factor of other secondary infections, as well.

1.2. Haptoglobin

Haptoglobin (Hp) is an acute phase plasma protein. Inflammatory cytokines induce the Hp expression causing elevated blood concentration. The molecule has potent anti-inflammatory and tissue repairing effect. Its main function is scavenging free hemoglobin (Hgb). Hgb is a prooxidant agent, induces the formation of reactive radicals that cause endothel and tissue injury and plays important role in oxidation of low density lipoprotein (LDL), so has atherogenic activity. Scavanging Hgb, Hp has a potent antioxidant effect. Following Hgb-Hp binding the oxidative activity of Hgb decreases, but does not disappear, so the clearance of the

complex is of importance. Hgb-Hp binds to special receptors, enters into cells, where it is metabolized.

Hp is synthesized primarily in the liver and is found predominantly in the blood, but small amount of it is present in extravascular sites. The molecule can sieve through vessel walls but granulocytes and monocytes are also capable of the de-novo synthesis of Hp. Extrahepatic form may act by autocrin and paracrin way and has regulatory functions in local inflammatory response. The main receptor of Hp is CD163 expressed on the cells of the monocyte-macrophage system, CD11b is found on granulocytes, natural killer cells, and on a small subpopulations of lymphocytes. Hp binds to CD22 on the surface on B cells.

CD163 is an endocytotic receptor of Hgb-Hp complexes that has bacterial sensor function, and has important role in initiating local immune response. In the second phase of inflammation it has regulatory function by reducing of cytokine expression. Connecting of Hgb-Hp complex to CD163 induces expression of interleukin (IL)-6 IL-10 and tumornecrosis factor-α (TNF-α). The induction of pro- and anti-inflammatory cytokines is different. The process is pushed to anti-inflammatory direction. The expression of CD163 is highest in the repairing phase of injury and inflammation, during tissue regeneration.

When binding to CD11b and CD22 haptoglobin may have a regulatory and immunemodulator effect and plays a role in the development of optimal immune response. In bone marrow binding to CD11b and CD22 haptoglobin influences the immune cell maturation. Hp-knock out mice have smaller immaturated B lymphocytes. Hp inhibits prostaglandin synthesis (adding further anti-inflammatory property) and has chaperon activity. So this molecule plays important role in host repair following inflammation and injury.

Haptoglobin polymorphism is related to two co-dominant allele variants (Hp1 and Hp2) on chromosome 16q22, which encodes the Hp α-chain, and this results in three major haptoglobin phenotypes (*Hp1-1, Hp2-1 and Hp2-2*). Homozygous Hp1-1 individuals express Hp*1-1* small homodimers. Homozygous Hp2-2 individuals express the Hp*2-2* phenotype, which consists of cyclic Hp polymers containing three or more monomers. The haptoglobin molecules synthesized in Hp2-1 heterozygous people are assembled into linear homodimers. In addition to structural variations, phenotype-dependent functional differences also exist. The clearance rate of Hp1-1 is greater than Hp2-2 though Hp2-2 complexes exhibit higher affinity to its receptors. Redoxactive iron in Hp2-2 is more available for the environment, than in Hp1-1, meaning a higher chelatable redox capacity.

Hp2-2 has a strong bacteriostatic function.

There is a distinct difference in antibacterial activity among phenotypes. Hp*2-2* acts as a bacteriostat by restricting access to iron, and causing agglutination. Large Hp*2-2* polymers are not suitable for bacteria to be engulfed as an iron source for their vital metabolic processes. The polymeric Hp*2-2* molecule also binds to surface proteins on the walls of streptococcus organisms and other bacteria, causing them to agglutinate thus markedly inhibiting their growth. The Hp*1-1* molecule is too small to cause agglutination and so does not have this inhibitory function. So polymer forms provide a natural defense against many pathogens. Binding to CD163 receptor they cause different types of cytokine induction. Hp1-1 pushes the response to Th2, while in case of Hp2-2phenotype Th1 response will be stronger

1.3 Mannose binding lectin (MBL)

MBL is a major pattern-recognition molecule, member of the C type lectin receptor family. It has multimer structure formed by multiple connection of one type protein chains. The structure has great importance in the function of the molecule, because it makes it capable to recognize the regularly repeated surface carbohydrate sequences of bacterial wall, and gives strong binding to pathogens. Following this binding a conformation change in MBL induces next steps (complement activation, phagocytic activation, inflammatory and adaptive immune response). In the circulation MBL is associated to MBL associated protein (MASP). The modification of MBL conformation causes the activation of MASP that cleave the complement-2 (C2) and C4, producing C4bC2a (the classic C3 convertase), so initiating the lectin way of complement activation. The stimulation of phagocytic function can happen not only through the activation of complement way. MBL works as an opsonin forming bridge through phagocytes and pathogens. The MBL has a role in the regulation of cytokine release. Low MBL level stimulates the synthesis of TNF- α, IL-1 β and IL-6, high concentration decreases them. Working as a TLR -2/ co-receptor, it has a role in the intracellular signaling, influences the phagocytic response given to the engulfed pathogen.
The molecule is synthesized mainly in the liver and is secreted into the bloodstream, but it can be found in extravascular sites and inside of phagocytes, too.
MBL coding MBL2 gene has more alleles that influence the serum level and activity of MBL. Homozygous missence mutation of the exon region cause very low MBL level (MBL deficient individuals). In one individual the MBL level and activity is genetically

determinated, it doesn't changes during the life as the consequence of inflammation, but the differences between individuals are great.

The occurrence of MBL deficiency is around 10-15% in general population. It has been reported as a risk factor for infections, particularly when immunity is already compromised through immunological immaturity, co-morbidity, or medical therapy. A significant correlation was reported between the risk of clinically significant post-transplant infections and the donor MBL deficiency in patients undergoing orthotopic liver transplantation. The occurrence of certain autoimmune diseases (ie. systemic lupus erythematosus [SLE]) was more common in MBL deficient individuals that can be explained by the delayed clearance of pathogens and apoptotic bodies.

1. AIMS

The two alleles of Hp α-chain coding gen form three different phenotypes: Hp1-1, Hp1-2 and Hp 2-2. They have different structure and size that affect their antioxidant and scavenger properties and they alter the immune responses to different directions. Phenotypes can change the course of some inflammatory diseases. We know little about that how the different immunmodulatory activity alters the appearance and course of bacterial infection in case of different phenotypes.

MBL is a major pattern-recognition molecule and an important component of the innate immune system. The molecule is able to recognize a wide range of common pathogens through their surface carbohydrate sequences and eradicate them. Upon binding to pathogens, MBL–in a complex with MBL-associated serin protease-2–mediates direct opsonophagocytosis and stimulates complement activation via the lectin pathway becomes the part of first line defense against bacterial invasion. MBL induces complement activation, activates phagocytosis and works as an opsonin. MBL deficiency has been reported as a risk factor for infections, particularly when immunity is already compromised through immunological immaturity, co-morbidity, or medical therapy.

Bacterial infections are a common cause of morbidity in patients with liver cirrhosis that can decompensate hepatic status and lead to death. Moreover, bacterial infections have been acknowledged as a potential trigger factor in many complications of liver cirrhosis, including variceal bleeding, hepatic encephalopathy, renal failure and impairment in clotting factors that alter the course of the disease. Advanced disease, reflected by Child-Pugh stage and the presence of gastrointestinal hemorrhage are the only sustainable and reproduced risk factors for bacterial infections. Multiple levels of immune dysfunction have been found in cirrhotic patients rendering them susceptible to bacterial infections. One of them is the opsonic disorder as the consequence of deficient complement system. Further decrease in opsonic function in case of MBL deficiency increases the susceptibility for bacterial infections.

Frequency of infections is different in patients with the same severity of liver cirrhosis. There must be some other risk factors out of known factors that influence the appearance of bacterial infection.

We conducted a follow-up observational study to find out whether the Hp polymorphism and the MBL are different in healthy and cirrhotic patients whether Hp polymorphism and MBL deficiency constitute risk factors for clinically significant infection in liver cirrhosis. Knowledge of new risk factors may allow for preventive interventions targeting at minimizing the infection-related mortality.

3. PATIENTS AND METHODS

3.1 Patients for Hp polymorphism

Serum samples were obtained from 336 consecutive cirrhotic patients with various etiologies (male/female [m/f]: 188/148, age: 56.5±10.8 years) at the Gastroenterology Division of 2nd Department of Medicine (Debrecen University) during the period from May 2006 to April 2008. The etiology of cirrhosis was chronic alcoholic intake in 219 cases (65.2%), hepatitis C virus-related in 97 (28.9%), and other causes in 20 (5.9%). The exclusion criteria were: evidence of gastrointestinal bleeding or bacterial infection in the preceding six weeks and prophylactic treatment with non-absorbable antibiotics in the preceding six months. The diagnosis of cirrhosis was based on clinical, biochemical, ultrasonographic, and, when available, histological features. Clinical data, including age, age at onset, etiology and severity of cirrhosis, presence and grade of ascites, encephalopathy, esophageal varices, prior episode(s) of variceal bleeding, and co-morbidities were collected. Myocardial infarction, congestive heart failure, peripheral arterial disease, cerebrovascular disease, chronic pulmonary disease, chronic renal failure, ulcer disease, diabetes mellitus, and non-metastatic and metastatic cancer, including hepatocellular carcinoma, were the co-morbidity diagnoses taken into account during data collection. Severity of cirrhosis was graded according to the Child–Pugh classification and the model for end-stage liver disease (MELD) score was calculated. After collecting the blood samples, patients were involved in a follow-up study until 1st of April 2009 or death/loss of follow up (median: 420 days [IQR: 88-742]) assessing the occurrence of clinically significant bacterial infections. Infections were considered clinically significant if they warranted hospital admission (n=719) due to deterioration of general condition or liver function and/or associated with complications, including variceal bleeding, hepatic encephalopathy, diuretic-resistant ascites formation, and renal failure.

The control group consisted of 384 age- and gender-matched healthy individuals (m/f: 192/192, age: 52.5 ± 10.2 years) selected from consecutive blood donors in Debrecen. Control subjects did not have any known gastrointestinal or liver diseases.

3.2 Patients for determination of MBL level

Five-hundred-ninety-one patients with various chronic liver diseases were investigated. Sera of patients with autoimmune liver diseases [ALD] (n=406, male/female ratio [m/f]: 122/184, age: 50.5 ± 17.0 years [yrs]: primary biliary cirrhosis [PBC, n=182], primary sclerotising cholangitis [PSC, n=76], and autoimmune hepatitis [n=148] or chronic hepatitis C [Chronic HCV, n=185, m/f:90/95, age 54.3 ± 12.5] were collected from six Hepatology Centers, five Hungarian (Debrecen University, Budapest Semelweis University, Pécs University, Miskolc Borsod-Abaúj Zemplén Country Hospital and Miskolc Szent Ferenc Hospital) and one German (Otto-von-Guericke University, Magdeburg). The diagnosis of PSC was based on biochemical evidence of cholestasis and the characteristic cholangiographic findings of bile duct stenosis and dilatations. In most cases, diagnosis was confirmed by compatible histology findings. The diagnosis of AIH was based on exclusion of other major causes of liver damage, including alcoholic, viral, drug and toxin-induced, and hereditary liver disease, and using the scoring system of the International AIH Group. The diagnosis of chronic HCV was based on positive HCV RNA, elevated liver function tests (>2x ULN for more than six month) and compatible liver biopsy, if available.

Serum samples were also obtained from 338 consecutive cirrhotic patients with various etiologies (m/f:189/149, age:56.4 ± 10.8 yrs) at the gastroenterology Division of 2nd Department of Medicine(Debrecen University) during the period from May 2006 to April 2008. The etiology of cirrhosis was alcoholic in 220 (65.1%), hepatitis C virus related in 98 (20.0%) and others in 20 (5.9%). The exclusion criteria and data evaluation were similar that in Hp examination.

3.3 Hp phenotype/genotype analysis

Hp phenotypes were determined with method established by Yang and coworkers based on sodium dodecyl sulfatepolyacrylamide gel electrophoresis of sera followed by

immunoblotting. Haptoglobin molecules were identified using 5%–10% gradient gels. After electrophoresis, proteins were blotted onto a Millipore polyvinyl-difluoride (PVDF) immobilion-P transfer membrane (Millipore, Bedford, MA, USA) by diffusion. The membrane was incubated with 1:1000 diluted polyclonal rabbit anti-human haptoglobin (Dako, Glostrup, Denmark). Following the wash with TBS-T (Towbin-Tween), the 1:2000 diluted goat anti-rabbit-HRP (horseradish peroxidase) antibody (Dako) was applied. Genotypes were reconstructed by comparison to Hp*1-1* and Hp*2-2* manufacturer standards (Sigma-Aldrich, Schnelldorf, Germany) run in each assay.

3.4 Determination of the MBL level

We used double-antibody sandwich ELISA system adopted from Michinton et al. to determine MBL levels. Microtiter plates (flat bottom, high binding capacity, Greiner Bio-One, Mosonmagyaróvár, Hungary) were coated for overnight incubation at 4^{o}C with monoclonal mouse anti-human MBL antibody (clone 131-1; BioPorto Diagnoses A/S Gentofte, Denmark). Three dilutions of sera (1/5, 1/25, 1/125) were then incubated for 90 min at 37 ^{o}C in a serial dilution of MBL standard (BioPorto Diagnosis A/S). A vial of standard solution was assigned an MBL content of 1000 AU and we accepted that it corresponded to 3200 ng/ml oligomerized MBL, as declared by the manufacturer.

3.5 Statistical methods

Continuous variables were summarized as means (standard deviation [SD]) or as medians (interquartile range or range [IQR]) according to their homogeneity. Categorical variables were compared with the χ^2-test or χ^2-test with Yates correction as appropriate. Continuous variables were compared with the Mann-Whitney U test or Student T test. Kaplan-Meier survival curves were plotted for analysis with LogRank and Breslow tests. Logistic regression analysis and forward stepwise Cox-regression analysis were used to assess the association between categorical clinical and laboratory variables and risk of and time to significant clinical infection. Associations are given as odds ratios (OR) or hazard ratios (HR) with a confidence interval (CI) established at 95%. A 2-sided probability value <0.05 was considered to be significant. For statistical analysis, SPSS15.0 (SPSS Inc, Chicago, IL) was used.

4. RESULTS

4.1 Hp phenotypes in cirrhotic patients

The Hp phenotype distribution of the cirrhotic patients was similar to that found in healthy control group. Hp1 and Hp2 alleles were in Hardy-Weinberg equilibrium and did not differ according to age or gender. In the 336 cirrhotic patients, a total of 248 infectious episodes were identified during hospitalizations. Approximately one third, 33.6%, of the patients presented some type of infections. Of the patients with an infection, 54.9% suffered from more than one episode. Distribution of types of infections was as follows: 32.5% urinary tract infection, 20.4% spontaneous bacterial peritonitis, 15.5% pneumonia, 8.7% skin and soft tissue infections, 3.4% bacteremia, 2.4% biliary tract infection, and 0.5% osteomyelitis. The origin of the infection could not be identified in 16.5% of the cases.

Bacterial cultures were positive in 138 (55.6%) of the infectious episodes. Bacteria were Gram-negative in 57.2% and Gram-positive in 42.8% of positive cases. The isolated bacteria were: *Escherichia coli* (23.2%), *Enterococcus faecalis* (15.9%), *Klebsiella pneumoniae* (15.2%), *Pseudomonas aeruginosa* (13.8%), *Staphylococcus aureus* (11.6%), *Streptococcus pneumoniae* (8.7%), *Proteus mirabilis* (2.9%), *Staphylococcus epidermis* (1.4%), others (7.2%).

There was no difference in the proportion of the localization or type of the bacterial infections among cirrhotic patients with the three different haptoglobin phenotypes. Of the clinical factors, the disease severity by Child-Pugh stage ($p=0.035$), the presence of ascites (OR: 2.45; 95%CI: 1.52-4.00, $p<0.001$), and co-morbidities (OR: 2.70; 95%CI: 1.70-4.30, $p<0.001$) were found to be risk factors for the development of clinically significant bacterial infections, as determined by using univariate analysis (χ2-test or χ2-test with Yates correction).

Hp phenotype was associated with the frequency of clinically significant bacterial infections. Patients with Hp*1-1* presented infectious episodes at a significantly higher rate than patients with other Hp phenotypes ($OR_{Hp1\text{-}1 \text{ vs. others}}$: 2.16, 95% CI: 1.07-4.33) as determined in a univariate analysis (χ2-test with Yates correction). However, there was no association between Hp concentration in quartiles and probability of developing infections. In a logistic regression analysis, the disease severity by Child-Pugh stage, presence of co-morbidities, and Hp phenotype were independent predictors of the development of infections.

In a Kaplan-Meier analysis, Child-Pugh stages, presence of ascites, and co-morbidities were associated with time to first systemic infection by Breslow and LogRank. A shorter time to first infection was found for Hp*1-1* patients, as compared to Hp*2-2* ones (median, 492 vs. 965 days; HR: 2.31 95%CI: 1.86-4.51, pBreslow=0.014 and pLogRank=0.017). In a Cox-regression analysis, Child-Pugh stage C (p<0.001), presence of co-morbidities (p=0.004), and Hp*1-1* phenotype (p=0.014) were independent variables associated with shorter time to first infection.

4.2 MBL levels and deficiency in liver diseases

The levels of MBL in patients with various chronic liver diseases and the control group were similar. Neither the MBL level nor the prevalence of MBL deficiency was statistically different between ALD, chronic HCV, and liver cirrhosis patients and was also comparable to the healthy controls. The occurrence of absolute MBL deficiency and low MBL level, as defined by serum level <100 ng/ml and <500 ng/ml, varied between 10.7%-15.6% and 31.1%-41.3%, respectively. MBL levels were significantly reduced in patients with the most advanced disease depicted either by Child Pugh stage or MELD score. However, the occurrence of absolute MBL deficiency was not different according to Child Pugh stage (Child A: 11.8%, Child B: 8.0%, Child C: 13.8%) or MELD score quartiles.

As the analyzed patient population was similar to the population examined for the Hp polymorphism, the results also were similar. In the 338 cirrhotic patients, a total of 251 infectious episodes were identified during hospitalizations. 33.7% of the patients presented some type of infection. Distribution of infections was the same we found in the firs part of the study. There was no difference in the proportion of the different type of bacterial infection between the absolute MBL deficient and MBL competent cirrhotic patients.

Absolute MBL deficiency (<100ng/ml) was significantly associated with the probability of developing infections in cirrhotic patients (OR: 2.15, 95%CI: 1.07-4.31, *p=0.039*). There was no association between these variables when the cut-off for MBL level was set at 500 ng/ml or when these results were re-analyzed according to MBL level quartiles (Q1:< 337, Q2: 338-1118, Q3: 1119-2454, Q4:>2455 ng/ml). In a logistic regression analysis disease severity by Child-Pugh stage, the presence of co-morbidities and absolute MBL deficiency were the independent predictors of bacterial infections.

Overall in-hospital mortality during the follow-up was 15% with 51 deaths occurring in the group of infected patients and 57 deaths in the patients without infection (20.3% vs. 12.1%, OR: 1.84, 95%CI: 1.22-2.78, p< 0.005). Absolute MBL deficiency was found to have an impact on the infection-related mortality. During the course of bacterial infection, risk for death was even higher in patients with absolute MBL deficiency (OR: 3.84, 95%CI: 1.29-11.37, p=0.013) using univariate analysis.

In a Kaplan-Meier analysis shorter time to first infection was found for absolute MBL deficient patients as compared to MBL competent ones (median, 579 vs. 944 days; HR: 2.05 95%CI: 1.10-3.83, *pBreslow=0.016* and *pLogRank=0.027*) .

A Cox-regression analysis for the development of bacterial infections showed that absolute MBL deficiency (*p=0.003)* besides Child-Pugh stage (*p<0.001*), and co-morbidities (*p=0.003*), and) were independent variables associated with shorter time to first infection.

5. DISCUSSION

We searched new risk factors of bacterial infections in liver cirrhosis besides the known two factors: gastrointestinal bleeding and severity of liver insufficiency. Selecting the most sensitive group and using antibiotic prophylaxis in this population the mortality rate can be decreased.

Investigating new molecules and their functions helps to understand the details of protection against bacterial invasion and to identify their clinical importance. We demonstrated that molecular variations having minimal importance in healthy volunteers can mean a severe risk factor in immune-deficient patients.

We chose two molecules that by the basic researches could have a role in the development of bacterial infections. The prevalence of absolute MBL deficiency (<100 ng/ml) and low MBL level (<500 ng/ml) in patients with various chronic liver diseases were similar to those observed in the control group that means it is not an important factor in the etiopathogenesis of liver diseases. We found no differences between MBL level and HP phenotypes in patients with cirrhotic and non cirrhotic liver diseases. Accordingly to earlier studies we could confirm that advanced liver cirrhosis (Child-Pugh C stadium) was an important predictor of bacterial infections. Severe co-morbidities also elevated the clinical risk and were independent risk factors by using multivariate analysis. Our data showed that in

presence of ascites the chance of bacterial infections was also higher. One explanation can be that peritoneal effusion means a more advanced disease. On the other hand we know that bacterial translocation is an important mechanism in development of bacterial infections in patients with liver cirrhosis. Animal and human studies confirmed, that bacterial translocation is more intensive in patients with ascites.

As the Hp molecule has phenotype dependent anti-inflammatory properties and exerts an immunomodulatory effect on innate and adaptive immune processes it was reasonable to assume that Hp polymorphism might be related to bacterial infectious episodes among patients with liver cirrhosis. During a follow-up study we found that the three Hp phenotypes influenced the development of infections with altered way. The first main finding of the present study was that the Hp*1-1* polymorphism was an independent predictor of risk for bacterial infection in patients with liver cirrhosis, and the time to first infection was shorter in patients with this phenotype. To our knowledge, this is the first report on the role of Hp genetic polymorphism in relation to bacterial susceptibility in a large cohort of patients with liver cirrhosis. The rapid worldwide spread of Hp2 allele in humans might be due to the ability of the Hp2 allele to provide selective resistance to foreign pathogens. As a consequence of molecule structure Hp*2-2* has a bacteriostatic function (by restricting access to iron and agglutination of bacteria) while Hp*1-1* has not. Hp*1-1* was also associated with weaker immune reactivity as indicated by lower B cell and CD4-T lymphocyte counts in the peripheral blood and stronger inhibitory effect on prostaglandin synthesis.

Th1 cellular immune response is essential to promoting elimination of the bacteria, Th2 to the limitation of inflammatory process, and tissue heeling. Hp*1-1* induces a Th2 type response. The function of Hp*2-2* changed with the structure. This form is weaker inducer of IL-6 and Il-10 shifting the T cell response towards dominant TH1. Dominant TH1 response has a favorable influence on bacterial infection and unfavorable on vascular complication of diabetes mellitus. Relative lack of TH1 processes would also be a reasonable explanation for the enhanced susceptibility to bacterial infections in individuals carrying Hp*1-1*. Balance of T cell response might have special importance in the extravascular space.

Large inter-individual variations were observed in serum haptoglobin concentrations of both the patients and the control subjects. Since haptoglobin is an acute phase protein that responds to inflammation, only subjects with normal CRP level were enrolled to avoid this confounding effect. Level of Hp is influenced by many factors. P.e. phenotypes (lowest level can be measured in case of Hp*2-1*.) or hemolysis. Synthesized by the liver, it is reasonable to assume that Hp level decreases parallel with the impairment of hepatic function. There are

data that show liver cirrhosis itself is an inductor of Hp synthesis. And during an infection cirrhotic liver maintains the synthesis of Hp at the expense of other metabolic processes. Certain co-morbidity also can induce Hp production (such as hepatocellulare carcinoma). We found no connection between the Hp concentrations at the inclusion time and appearance of infections during the follow up period. One explanation can be that circulating Hp is synthesized mainly by the liver but in controlling innate and adaptive immune response Hp produced locally by granulocytes and monocytes play more important role. This synthesis is independent of liver capacity.

These findings add to the literature on the impact of the Hp molecule in host defense and call for additional basic research on the role of Hp in innate and adaptive immunity.

In the second part of the study we investigated the complex associations between MBL level and the disease etiology along with severity in a large cohort of patients with various chronic liver diseases. To our knowledge, this is, to date the largest study to investigate this connection. Levels of MBL is determinated genetically, Mutations of *MBL2* coding gen: homozygotes or compound heterozygotes have profoundly reduced MBL levels. Due to the additional effects of non-coding polymorphisms and other unknown factors, the individual MBL values, however, vary substantially in wild-type homozygotes and mutation heterozygotes, thus providing only a rough guide to serum MBL concentration (that is high level and intermediate level, respectively). These genetically non-MBL-deficient groups also include a significant number of serologically MBL-deficient individuals

In the present study, MBL levels in cirrhotic patients of various etiologies were similar to those ones in non-cirrhotic liver groups. In a more detailed analysis of this group, however, MBL levels were significantly lower in advanced liver diseases, (median values, Child C: 716 ng/ml vs. Child A or B: 1444 or 1375 ng/ml, $p<0.001$ for both). These results are not surprising, as the synthetic function of the liver decreases with the progression of the cirrhosis. At the same time occurrence of absolute MBL deficiency was not more frequent in Child C patients, implying a genetically-based predominance in the background.

MBL is a C-type serum lectin that plays a central role in the innate immune response. As recently found, MBL functions as a TLR co-receptor that enables the molecule to spatially coordinate, amplify, and synchronize the innate immune defense system. When the adaptive immune response is immature or compromised, the innate immune system constitutes the principle defense against infection. Clinical studies have shown that MBL deficiency predisposes immune compromised patients to bacterial infections. Liver cirrhosis has been characterized as an acquired immunodeficiency syndrome. Thus, it is reasonable to assume

that the MBL deficiency might be related to the bacterial infectious episodes among patients with liver cirrhosis. The main finding of our follow up study was that the absolute MBL deficiency was an independent predictor of the risk for bacterial infection, and the time to first infection was also shorter in patients with absolute MBL deficiency.

Further deterioration of local immune defense in the gut would also be of significance in cirrhotic patients with MBL deficiency. Extrahepatic transcription of *MBL2* gene has been reported in the small intestine. Though the mRNA level comprises about 1% of that seen in the liver, the inflamed gut is, in fact, able to increase MBL synthesis. Transcription of the *MBL2* mRNA was found to be highly upregulated in the inflamed intestinal tissue samples. The *MBL2* gene is expressed in the immune cells infiltrating the inflamed gut. Significant intracellular and minor surface expressions of the molecule have also been described in adherent human monocytes and monocyte-derived dendritic cells.

The presence of absolute MBL deficiency (similar to Hp*1-1* phenotype) was an independent predictor for the risk of and shorter time to develop bacterial infections in liver cirrhosis, suggesting that this is a surrogate marker of the clinical risk assessment, in addition to Child-Pugh stage and co-morbidities. These findings further emphasize the impact of the MBL molecule in the host defense in an immune compromised state.

In conclusion, the Hp phenotype and the MBL levels in patients with chronic liver diseases were not different from those in healthy subjects; however, MBL level was lower in advanced liver cirrhosis. Hp*1-1* phenotype and absolute MBL deficiency was not associated with any type of chronic liver diseases or with the presence of liver cirrhosis but they were independent predictors for risk of bacterial infections. We demonstrated that control role of MBL and Hp molecules in innate immunity has clinical importance. We found two new predictors for risk of bacterial infections in liver cirrhosis in addition to the known two factors: gastrointestinal bleeding, and severity of liver disease.

To clarify the exact importance of Hp and MBL needs further resources.

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List of publications related to the dissertation

1. **Vitális, Z.**, Altorjay, I., Tornai, I., Palatka, K., Kacska, S., Pályu, E., Tornai, D., Udvardy, M., Hársfalvi, J., Dinya, T.: Phenotypic polymorphism of haptoglobin: A novel risk factor for the development of infection in liver cirrhosis.
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2. Altorjay, I., **Vitális, Z.**, Tornai, I., Palatka, K., Kacska, S., Farkas, G., Udvardy, M., Hársfalvi, J., Dinya, T., Orosz, P.: Mannose-binding lectin deficiency confers risk for bacterial infections in a large Hungarian cohort of patients with liver cirrhosis
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List of other publications

3. Papp, M., Norman, G.L., **Vitális, Z.**, Tornai, I., Altorjay, I., Földi, I., Udvardy, M., Shums, Z., Dinya, T., Orosz, P., Lombay jr., B., Par, G., Par, A., Veres, G., Csak, T., Osztovits, J., Szalay, F., Lakatos, P.L.: Presence of Anti-Microbial Antibodies in Liver Cirrhosis: A Tell-Tale Sign of Compromised Immunity?
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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

Zsuzsanna Vitális is considered as a first author in case of article No. 2 because the author contributed equally to the work.

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