**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)**

# Evaluation of the humoral components of innate and adaptive immunity in gastroenterological diseases with chronic inflammation


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**UNIVERSITY OF DEBRECEN**

**KÁLMÁN LAKI DOCTORAL SCHOOL**


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**EVALUATION OF THE HUMORAL COMPONENTS OF INNATE AND ADAPTIVE IMMUNITY IN GASTROENTEROLOGICAL DISEASES WITH CHRONIC INFLAMMATION**

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The Examination takes place at the Library of the Department of Pediatrics, Faculty of Medicine, University of Debrecen, 19th of June, 2017. 11:00 am

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen 19th of June, 2017. 13:00 pm

# INTRODUCTION

The basis of maintenance of immune homeostasis is the close collaboration between the elements of innate and adaptive immunity. The humoral (complement system, antimicrobial peptides, cytokines) and cellular (phagocytes, natural killer (NK) cells and dendritic cells) components of innate immunity constitute the first line of immunological defense against intruder pathogens and they play a key role in triggering adaptive immune response. The humoral (cytokines, antibodies) and cellular elements (T and B lymphocytes) of adaptive immune response are activated following a certain latency period. However, the two defense systems do not function independently of each other, but they provide protection of the host organism in an intertwined way. The increased reactivity or reduced functioning of the immune system plays a key role in the pathogenesis of several gastroenterological diseases. In the course of our clinical research, we selected two gastroenterological patient groups (coeliac patients and cirrhosis patients) in which the dysfunction of the innate immune system plays a significant role in the pathogenesis of the disease and the developed complications. It is the common characteristic of the two diseases that the inflammation and structural and functional impairment of the small intestine walls lead to increased permeability, which, coupled with the altered bowel flora, results in chronic bacterial translocation. The presence of various bacteria and the immune response developed against them may affect the severity of local or systemic inflammation.

## Coeliac disease

Coeliac disease (CD, gluten-sensitive enteropathy, sprue) is an autoimmune enteropathy triggered by the gliadin component of gluten and mediated by the T cell, developed in genetically susceptible patients which also shows extraintestinal manifestation.

The development of the disease is very diverse, there is little to be known about which factors affect symptom types. The clinical manifestation of the disease is either classic, when the signs and symptoms of malabsorption (diarrhoea, weight loss, steatorrhoea and growth failure) can be easily detected, or non-classic (gastrointestinal symptoms and/or extraintestinal manifestations) and asymptomatic. During the recent years, increasing number of non-classic and asymptomatic patients were diagnosed worldwide.

The disease is characterised by the formation of several antibodies (e.g. anti-transglutaminase antibodies: TGA; anti-endomysium antibodies: EMA, anti-gliadin antibodies, anti-deamidated gliadin peptide antibodies), even though the mechanisms in the background of

their generation and their specific role in the pathogenesis of the disease is not entirely known. The firstly detected anti-gliadin antibodies and the subsequently detected anti-reticulin antibodies are not used in CD diagnostics anymore due to their low sensitivity and specificity. Most recommendations refer to anti-TG2 and EMA. Currently, anti-synthetic deamidated gliadin peptide antibodies seem to be a viable option in CD diagnostics in certain cases.

The antibodies generated against the carbohydrate epitopes of the cell walls of various bacteria are markers of complicated Crohn's disease. The more frequent appearance of the anti-*Saccharomyces cerevisiae* antibody (ASCA) was described also in coeliac disease. At the same time, the frequency of the appearance and clinical CD-related significance of other antimicrobial antibodies detected in IBD (ALCA, AMCA, ACCA, anti-OMP) is still not clarified.

**Antimicrobial antibodies**

Glycans are oligosaccharides consisting of identical monosaccharide units linked together with glycosidic bond. Glycans are present in the cell surfaces of red blood cells, immune cells and various microorganisms. Furthermore, these oligosaccharides are important molecules of the humoral and cellular immune system, as they play a key role in developing links between cells, as well as in the innate immune response and the antibodies generated against them detect the antigens consisting of the carbohydrate components on the surface of pathogens. This phenomenon explains why anti-glycan antibodies are considered to be a possible biomarker for inflammatory and autoimmune diseases. The anti-mannobioside [Man(α1.3) Man(α)] carbohydrate epitope IgG antibody or AMCA IgG belongs to the group of anti-mannan antibodies. Anti-laminaribioside [Glc(β1.3)Glc(β)], and anti-chitobioside [GlcNAc(β1.4)GlcNAc(β)] antibodies (ALCA IgG and ACCA IgA) are also anti-glycan antibodies. The anti-OmpC/anti-OMP IgA antibody is generated against the transport protein porin C in the outer membrane of *Escherichia coli*.

The most frequently examined anti-mannan antibody is the anti-*Saccharomyces cerevisiae* antibody (ASCA IgG and IgA or gASCA), which is generated against the oligomannose structure in the cell wall of *Saccharomyces cerevisiae* that is found in bakers' yeast and brewers' yeast. As regards ASCA and other antibodies generated against microbial antigens, it can be stated that the exact mechanism and the significance of their development is not yet clarified. It is not known whether these antibodies have any role in the immune pathogenesis of inflammatory gastroenterological diseases [e.g. inflammatory bowel disease (IBD) or coeliac

disease] or their appearance is only the consequence of inflamed, permeable intestinal mucosa. Furthermore, the anti-microbial antibodies might represent genetic susceptibility because patients who have positive antibodies often carry mutations in the NOD2/CARD15 gene.
Anti-microbial antibody formation has also been reported in CD. ASCAs remain the most widely investigated antibodies in this patient group but increasing experimental data are available on newly discovered antibodies such as anti-I2 or anti-OmpW (Bacteroides caccae TonB- linked outer membrane protein).

**Cirrhosis**

The various factors impairing liver cells (most often alcohol consumption, viral infection, non-alcoholic steatohepatitis or autoimmune processes) result in typical histological lesions (necroinflammation, fibrogenesis) in the liver. As a result of the progression of these processes, it usually takes years or decades to develop definitive cirrhosis. In cirrhosis patients, the frequent reasons of the deterioration of clinical conditions are infections which have a fundamental role in the development of several complications, including variceal bleeding, hepatic encephalopathy, kidney failure and coagulation disorders. The development of bacterial infection quadruples the risk of mortality. 30% of patients die within a month following infection and another 30% die within one year. The prevention of bacterial infections is especially significant; therefore, it is necessary to early detect patients who run a great risk of such episodes. For a long time, only a few clinical forecast factors are known such as the Child-Pugh score (advanced stage and gastrointestinal bleeding). Cirrhosis patients are especially susceptible to bacterial infections and cirrhosis is one of the most frequent adaptive immune deficiency. The background of this phenomenon is the mechanisms affecting innate and adaptive immunity which are jointly referred to as cirrhosis-associated immune dysfunction (CAID).
The intestinal mucosal barrier in cirrhosis patients is impaired for various reasons, resulting in pathologic bacterial translocation and the amount of immunogenic bacterial products increases in the circulation. These products increase the pro-inflammatory processes which lead to the impairment of the liver tissue and the insufficient functioning of the soluble and cellular receptors, which play an active role in detecting bacteria, may result in increased susceptibility to infections.

### Complement system – lectin pathway

The complement system is an important element of innate immunity. The complement system can be activated in three ways: the classic, alternative and lectin pathway. The lectin pathway is activated by the binding of carbohydrates on the surface of MBL or ficolins and pathogens. Most molecules of the lectin pathway are generated in the liver and they have a key role in the natural defense of the host organism against pathogens. Mannose-binding lectin, ficolins (FCN) and collectins act as soluble pattern recognition molecules (sPRM), while mannose-binding lectin associated serine proteases (MASP) are the effector molecules of pathogen elimination. So far, human analyses led to the identification of three ficolins: L-ficolin (**FCN-2**), H-ficolin **(FCN-3)** and M-ficolin **(FCN-1)**. The main activator of the system is MASP-2 which creates a complex with MBL, H-ficolin and L-ficolin and circulates in the plasm. MASP-2 is activated if MBL or ficolins are bound to the pathogen-associated molecular pattern. As a next step, MASP-2 is able to split C2 and C4, resulting in the C3 convertase C4bC2b.

The low level of functional proteins increases the risk of various infections, mainly in the case of diseases associated with immunosuppression. However, the role of functional proteins in bacterial infection is less known.

## OBJECTIVES

### Coeliac patient group

We hypothesized that newly discovered inflammatory bowel disease (IBD)-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in CD and aimed to determine the prevalence of these antibodies in a Hungarian cohort of adult CD patients in relation to clinical presentation, GFD and NOD2/CARD15 mutations.

### Cirrhosis patient group

In this study, we aimed to assess the serum levels of various lectin pathway molecules in a large cohort of stable outpatients with cirrhosis and also their association with the disease specific characteristics. In addition, in a 5-year follow-up observational study, we aimed to evaluate whether serum levels of various lectin pathway molecules constitute a risk for the development of cirrhosis-associated bacterial infections.

# PATIENTS AND METHODS

## Patient groups

### Coeliac patients

One-hundred and ninety consecutive, unrelated Hungarian adult patients with biopsy-proven CD (male/female: 71/119, mean age: 39.9 years, SD: 14.1) and 66 of their first degree relatives (siblings, mean age: 37.7 years, SD: 13.9) were investigated. The diagnosis of CD was based on small bowel biopsy showing severe villous atrophy with crypt hyperplasia (Marsh type III lesions) and elevated serum levels of antibodies against transglutaminase (TGA) and endomysium (EMA).

Of the 190 patients, 82 patients' sera were obtained at the time of diagnosis (Group CD1) and in 30 of these 82 patients further serum samples were re-evaluated for the same antibodies after adherence to long-standing GFD. The median follow-up period between these blood samplings was 28.5 mo [interquartile range (IQR): 18-52]. In the 108 remaining cases the diagnosis of CD had been established prior to this study and they adopted a strict GFD. These 108 patients were divided into two separate groups according to their current TGA and EMA status and dietary compliance at the time of the sampling. Thirty-three patients still had positive EMA and TGA results (Group CD2) and the median duration was here 3.5 mo (IQR: 1-11). The adequate compliance was indicated by reduced antibody titers as compared to those at diagnosis. The remaining 75 patients had negative EMA and normal TGA titers (Group CD3), median follow up: 21 mo (IQR: 6-85). Detailed clinical data concerning the clinical presentation of CD at diagnosis were classified as follows: (1) severe generalized malabsorption; (2) non-specific gastrointestinal symptoms that did not compromise the general condition; (3) iron deficiency anemia without major gastrointestinal complaints; (4) dermatitis herpetiformis; (5) silent disease (population screening); (6) other (autoimmune diseases, reduced bone mineral density, liver disease, brain disease). Patients were assigned to one of these major presentation types in a prospective manner, based on clinical and routine laboratory results at diagnosis.

The control group consisted of 100 healthy, ethnically similar, blood donor individuals (male/female: 47/53, mean age: 36.6 years, SD: 9.1) who had normal findings on a thorough medical examination, blood pressure measurements, and routine laboratory tests. A second non- celiac gastrointestinal disease control group consisted of 48 patients with irritable bowel syndrome/diverticulosis without inflammation (male/female: 21/26, mean age 40.4 years, SD:

16.1). Further comparisons were performed with the previously published Crohn's disease patient group in which the same antibodies were analysed.

**Cirrhosis patients**

The patients involved in this study constitute a part of the subgroup of 404 patients who were examined during regular or special follow-up visits in outpatient practice or due to acute decompensation (AD) episodes in hospital treatment between 1st May 2006 and 31st December 2010. Serum samples of 378 patients (266 outpatients and 117 hospitalised patients due to AD episodes) were accessible for the analysis. In the course of a subsequent serological evaluation, we involved stable outpatients into the study, while AD patients were excluded. According to the usual criteria, one or more combinations of the following events were defined as acute decompensation: generation of a large quantity of ascites (stage II/III), acute hepatic encephalopathy, acute gastrointestinal bleeding and/or systemic bacterial infection. Refractory ascites patients were not involved either.

Medical documentation containing clinical data was retrospectively analysed for the period prior to the observational follow-up study. At enrolment, disease severity assessed by liver-oriented scores (Child-Pugh and MELD) and clinical stage of the diseases (compensated/decompensated) was determined.

We involved patients in the follow-up study whose AD time and type which confirm their hospital admission were registered by the gastroenterologist. The follow-up period lasted for 5 year or death/exclusion from follow-up. 85 patients (32%) died during follow-up, with the median period until death being 656 (IQR: 277-971) days. The median follow-up period of the 181 surviving patients was 1107 (IQR: 411-1825) days.

The definition of the appearance of clinically significant bacterial infections (spontaneous bacterial peritonitis, urinary tract infection, pneumonia and other infections of the skin and soft tissue, as well as biliary, mouth and digestive tract infection, osteomyelitis, endocarditis, bacterial infection of unknown origin without location-specific infection in the case of positive haemoculture) was based on carefully performed clinical, laboratory and imaging examinations.

The healthy control group consisted of 160 age- and gender-matched individuals (male/female: 72/88, age: 51.5±16.9 years) selected from consecutive blood donors in Debrecen. The control subjects did not have any known gastrointestinal or liver diseases.

## METHODS

***Identification of antimicrobial antibodies***

We used serum samples obtained from peripheral blood which were stored at -80ºC until use. The presence of antimicrobial antibodies was determined with the ELISA method, using commercially available kits in accordance with the instructions of the manufacturer: gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA (IBDX®, Glycominds Ltd., Lod, Israel), ASCA IgG, ASCA IgA and OMP IgA (QUANTA Lite™ OMP PLUS ELISA, INOVA Diagnostics, San Diego, CA). The results were presented as arbitrary units, which were calculated based on sample and calibrator optical density. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60 and 90 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, respectively, and 25 U/mL for anti-OMP.

***Detection of NOD2/CARD15 mutations***

One-hundred and thirty-four CD patients and 100 healthy control subjects were eligible for NOD2/CARD15 mutation analysis. Major NOD2/CARD15 mutations (SNP 8, 12 and 13) were determined by denaturing high-performance liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic Limited, UK). Sequence variation, observed in the dHPLC profile, was sequenced on both strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA)

***Examination of the lectin system***

Blood samples were obtained at enrolment from each patient and were frozen at -70°C until testing. Serum level of MASP-2 was determined using the ELISA method, according to the manufacturer's instructions (Hycult Biotechnology, Uden, Netherlands). Samples were measured in duplicates on the same plate, and the mean values were used. Between runs, coefficients of variation (CV) were 8%. The limit of detection was 1.6 ng/mL. According to literature data, serum level of <100ng/ml was considered as MASP-2 deficiency.

We set up a sandwich ELISA system to determine serum level of L-ficolin (FCN-2) and H-ficolin (FCN-3). Briefly, microtiter plates (flat bottom, high binding capacity, Greiner Bio-One Hungary, Mosonmagyarovar, Hungary) were coated for 6 hours at 37°C with 0.75 μg/ml monoclonal mouse anti-human Ficolin-L (clone GN4) and Ficolin-H (clone 4H5) antibodies (HyCult Biotechnology) in carbonate/bicarbonate buffer (pH 9.6). Different dilutions of the sera (1/10 for Ficolin-L, 1/1000 for Ficolin-H) were then incubated in duplicates for overnight at +4°C in a wet chamber together with a serial dilution of Ficolin-L and Ficolin-H standards

(recombinant human Ficolin-L and Ficolin-H; R&D Systems Inc., Minneapolis, MN, USA). Serum standards concentrations ranged from 2.3 ng/ml to 150 ng/ml for Ficolin-L and from 0.8 ng/ml to 50 ng/ml for Ficolin-H and were included in each run. After washing three times, biotinylated polyclonal goat anti-human Ficolin-L and Ficolin-H antibodies (R&D Systems Inc., Minneapolis, MN, USA) – 0.15 μg/ml concentration in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) at a pH 7.4 – were added for 2.5 hours at room temperature and followed by another washing step. Avidin–biotinylated-peroxidase-conjugate (Vectastain, Vector Laboratories Inc., Burlingame, CA) at 1:1000 dilution was added and incubated for 30 min at room temperature in a wet chamber. Color was developed with tetramethyl-benzidine dihydrochloride (TMB; Sigma–Aldrich, Schnelldorf, Germany), stopped with 2M H2SO4, and read immediately at 450 nm in a Labsystem Multiscan MS plate reader (Thermo Scientific, Budapest, Hungary). The calculation of results was performed using the Genesis software program with four parametric logistic curve fitting.

In the case of duplicated samples, if the coefficients of variation (CV) were >20%, we repeated the measurement. The CV was 17% for FCN-2 and 14% for FCN-3 in between runs. Since no definition exists for FCN deficiencies in the present study, low level of FCN-2 (<427ng/ml) and FCN-3 (<4857ng/ml) were determined arbitrary corresponding to the 25th serum level percentiles of the patients.

**Statistical analysis**

The distribution of constant variables was analysed using the Shapiro-Wilk W test. The data was presented in accordance with the distribution in the form of median (percentile range: 25-75), mean ± standard deviation and number of cases (%).

*t*-test with separate variance estimates, $\chi 2$-test, $\chi 2$-test with Yates correction and likelihood ratio (LR) test were calculated to evaluate differences between various groups of CD patients and controls, as well as within subgroups of CD patients, as appropriate. Sensitivities, specificities, positive and negative likelihood ratios were calculated to determine the predictive power of gASCA, AMCA, ALCA, ACCA, OMP or the combination of these markers in distinguishing between CD and controls. Spearman's rank order correlation was calculated to test the association between anti- glycan/OMP and TGA levels. Two-sided t-test for independent samples with separate variance estimates and ANOVA with post hoc Scheffe test were used to analyse the association between anti-glycan antibody titers and clinical symptoms at diagnosis. A P value of <0.05 was considered as significant.

Categorical variables were compared using Fisher's exact test or $\chi 2$test with Yates correction, as appropriate. Continuous variables were compared with Mann-Whitney U test or Kruskal-

Wallis H test with Dunn's multiple comparison post hoc analysis. Paired samples were analysed by Wilcoxon signed rank test. The Spearman's nonparametric rank correlation test was used to determine correlations.

The association between categorical clinical variables or serum levels of lectin pathway molecules and adverse disease outcomes during follow-up were assessed by Kaplan-Meier analysis and univariate Cox-regression analysis. The significance of subgroups was evaluated with log-rank test. Multivariate analyses were performed with backward elimination procedure and likelihood ratio test to identify independent risk factors. Associations are given as hazard ratio [HR] with 95% confidence intervals [CI].For statistical analysis and graphical presentation the SPSS 22.0 [SPSS, Chicago, IL], and GraphPad Prism 6 [San Diego, CA] programs were used. A 2-sided probability value of $<0.05$ was considered to be statistically significant.

## RESULTS

### Analysis of antimicrobial antibodies in coeliac disease

The frequency of gASCA IgG, AMCA IgG, and ACCA IgA was significantly higher at the time of diagnosis of CD than in healthy and non-celiac gastrointestinal control groups. However, the frequency of ALCA IgG and anti-OMP positivity in the patients was similar to those in control groups. No difference was found between healthy subjects and GI controls based on the presence of these antibodies. For that reason, we only used the healthy subjects as a control group in the subsequent comparisons. When calculating the sensitivity and specificity of the different markers based on the cut-off values suggested by the manufacturer, 65.9% of the CD patients were positive for at least one of the tested anti-microbial antibodies at the time of diagnosis. Except ALCA, all anti-glycan and anti-OMP antibodies were specific for untreated CD. However, the overall sensitivity was low (gASCA: 39.0%, AMCA: 35.4%, ACCA: 37.8%). Compared to healthy controls, gASCA, AMCA, and ACCA were associated with a moderate increase in the likelihood of CD, respectively. The positivity of any anti- glycan antibody significantly increased the likelihood for untreated CD.

Detailed clinical data on the symptoms at the time of diagnosis in Group CD1 was available in 78 patients out of 82. Of the 78 patients, 32 (41%) presented with severe malabsorption, 34 (43.6%) with non-specific or minor gastrointestinal symptoms, 9 (11.5%) with iron deficiency anemia, and 3 (3.9%) with other symptoms. The titers of the anti-glycan antibodies varied according to the presenting symptoms. Patients with severe malabsorption more frequently had multiple antibodies ($P = 0.019$) while in those with non- specific gastrointestinal symptoms or iron deficiency anaemia no seroreactivity or reactivity against only one glycan

components was more commonly seen (Table 4). Out of the CD patients with multiple antibodies positivity, 65.4% were diagnosed because of malabsorption, which was significantly higher than in CD patients with another serotype group (0 = 26.9%, or 1 = 34.8%, P = 0.019). Significant correlation was observed between the anti-glycan antibodies and the TGA level (pgASCA < 0.001, R = 0.39; pAMCA = 0.01, R = 0.28; pALCA = 0.006, R = 0.23; pACCA < 0.0001, R =0.53; p antiOMP = 0.001, R = 0.25, using Spearman's correlation test). Similarly, positive correlation was found between EMA IgA and gASCA ($p < 0.001$), AMCA ($p < 0.001$), ACCA ($p< 0.0001$), or any anti-glycan ($p < 0.0001$) antibody, while there was no correlation in the case of anti-OMP.

In the group of 30 patients who were evaluated both at diagnosis and following a long term GFD (subgroup of Group CD1), initial positivity for anti-glycan antibodies (gASCA in 12, AMCA in 9, and ACCA in 11 patients) observed at diagnosis was lost after GFD. The titer of each antibody decreased significantly after adherence to GFD ($P < 0.001$ for each). Anti-OMP antibody positivity behaved similarly, with all but one of 14 patients positive at diagnosis becoming negative after GFD, but the titer significantly decreased also in the case of this patient during the 135-day-long GFD.

The prevalence of NOD2/CARD15 mutation in CD (19/134, 14.2%) did not differ from that in the control group (16/100, 16%). Additionally, we did not observe any association between symptoms at presentation or anti-glycan antibody positivity and the presence of NOD2/CARD15 variants.

**Analysis of lectin pathway molecules in cirrhosis patients**

FCN-2 and FCN-3 levels were significantly lower in cirrhosis patients than in the control subjects (FCN-2: 505[426–596] vs 769 [629–1145] $p<0.001$ and FCN-3: 7301[4857–10601] vs 10797 [9017–13867] $p<0.001$). The MASP-2 level was also significantly lower (MASP-2: 212[126–359] vs 412 [285–586]), while MASP-2 deficiency (<100 ng/ml) was also more frequently observed in cirrhosis patients (19.5% vs 2% $p<0.001$).

In cirrhosis, levels of all three lectin pathway molecules decreased gradually according to disease severity, as rated by the Child-Pugh stage. Furthermore, both types of the FCNs but not the MASP-2 levels were significantly lower in patients with ascites as compared to those without.

FCN levels were also associated with disease etiology to some degree. Both types of the FCNs levels were significantly lower in alcoholic as compared to non-alcoholic patients, but only in patients with Child A stage (FCN-2: 505 [440 – 562] vs. 548 [489 – 679] ng/ml, p=0.001 and

FCN-3: 7509 [5508 – 10897] vs. 10596 [7065 – 15379] ng/ml, p<0.001, respectively), or in those without ascites (data not shown). No similar association was found in the case of MASP-2.

Significant correlation was found between levels of lectin pathway molecules and laboratory markers of impaired renal and liver function and accordingly with liver-oriented scores (Child-Pugh and MELD) or with the laboratory parameters of portal hypertension.

The sequential changes of serum lectin levels were observed in the subgroup of stable outpatients (n=65). As a first step, the serum samples of 33 patients were analysed during subsequent outpatient follow-ups (655 [IQR: 246-1090] days passed between two samplings). No significant change was found in the serum level of either examined molecule (FCN-2: 504 [458-600] vs. 479 [423-543], p=0.092; FCN-3: 9449 [5934-13070] vs. 8814 [5999-11006], p=0.837; MASP-2: 221 [129-369] vs. 208 [119-324], p=0.313). During this period, no significant change was observed between the different Child-Pugh stages either (p=1.000, using the Wilcoxon rank sum test).

Lectin level measurements were performed in a different subgroup of 32 patients at the time of the AD episode. No significant difference was observed in the level of either molecule (FCN-2 164 [107-284] vs. 178 [102-314], p=0.772; FCN-3: 6455 [3877-9204] vs. 5978 [3723-8751], p=0.400; MASP-2: 183 [106-329] vs. 167 [78-276], p=0.140). The median period between two samples was 555 days [133–955].

**Significance of serum levels of lectin pathway molecules in the risk of clinically significant bacterial infections**

Ninety-five (35.7%) of involved patients encountered at least one episode of CSI during the follow-up period. The median time to the development of first infection was 626 [169-799] days. Urinary tract infection was the most commonly diagnosed CSI and accounted for 33.7% of events, followed by spontaneous bacterial peritonitis (24.2%) and pneumonia (12.6%). 5.3% of the cases were multifocal. Other detected bacterial infections included erysipelas (4.2%), cholangitis (2.1%), bacteriaemia (2.1%) and acute bronchitis (3.2%), while the infection could not be located in 12 cases (12.6%). As regards the serum level of the lectin pathway molecules, it can be stated that FCN-2 and FCN-3 levels were lower than normal molecular levels and they correlated with the increasing cumulative incidence of clinically significant bacterial infections (FCN-2: 62.6% vs. 46.7% HR: 1.55, 95%CI: 1.00-2.39, p=0.047 and FCN-3: 59.3% vs. 48.2% HR: 1.61, 95%CI: 1.05-2.47, p=0.029)

The serum ficolin profile, in the case of which both ficolins' molecular levels were taken into

consideration simultaneously, showed a gradually increasing cumulative incidence in relation to the development of clinically significant bacterial infection, with the increase of the number of low ficolin levels: 45.7% (both ficolins at normal level), 57.2% (if one of the two ficolins was at a low level) and 63.8% (both ficolins at low level) (HR: 2.00, 95%CI: 1.15-3.47, p=0.016). No similar correlation was found either in the case of individual MASP-2 deficiency or MASP-2 deficiency combined with ficolins.

The univariate Cox regression analysis of clinical factors related to the development of clinically significant bacterial infections revealed that alcoholic disease etiology (HR: 1.68, 95%CI: 1.07-2.66, p=0.024), advanced disease stage (Child-Pugh stage B/C [HR: 2.42, 95%CI: 1.61-3.64, p<0.001] or presence of ascites [HR: 2.31, 95%CI: 1.54-3.46, p<0.001]) and prior CSI episode (HR: 2.64, 95%CI: 1.76-3.96, p<0.001) were significantly associated with the increased risk for the development of CSI. However, the multivariate Cox regression analysis, which involved both the serum levels of lectin pathway molecules and the respective clinical variables, showed that only the clinical factors such as advanced disease stage and prior CSI episodes were proved to be independent risk factors in terms of the development of clinically significant bacterial infections, but this was not the case either in relation to each ficolin or the ficolin profile.

We observed the potential effect of serum ficolin profile and MASP-2 deficiency on infection-related mortality (n=95). 19 patients (20%) died during the first bacterial infection. According to the Kaplan-Meier survival test, neither the ficolin serum detected at the time of recruitment, nor the MASP-2 deficiency was in correlation with the risk of mortality. There were 85 cases of death related to liver diseases in the whole cirrhosis patient group (32. %). The Kaplan-Meier survival test showed that the chance of survival is significantly lower in the case of patients suffering from a more advanced stage of disease based on the Child-Pugh score (p<0.001), those who had already suffered from ascites (p<0.001) or prior CSI episodes (p=0.035). The MASP-2 deficiency (p =0.387) or the serum ficolin profile (p =0.093) was not in correlation with survival altogether.

## DISCUSSION

### *Evaluation of antimicrobial antibodies in coeliac disease*

This is the first report to investigate the complex associations between a panel of new serological markers, clinical presentation of the disease, and NOD2/CARD15 status in a relatively large cohort of CD patients. The examination of antimicrobial antibody generation in coeliac patients can potentially be a new element of observing antibody generation.

In this study, we demonstrated that the presence of anti-glycan antibodies (gASCA, ACCA, and AMCA) are associated with CD at the time of diagnosis. However, the prevalence of ALCA and anti-OMP did not differ from the results in the control group. The rate of gASCA positivity (39%) at the time of diagnosis of CD was comparable to the results in CD patients in previous studies.

Current data advocate that in both CD and Crohn's disease patients have a primary defect in intestinal permeability that is also shared by a subgroup of relatives. In CD, it is also apparent that the exposure to gluten results in mucosal inflammation and the consequent tissue damage further abrogating the primary gut barrier defect, while gluten removal resolves the enhanced intestinal permeability.

Comparing the data of coeliac patients with our own IBD patient population, no significant difference was noted between the two groups in terms of the prevalence of ASCA IgG (39% vs 50.5%, P = 0.091). In the present study, except ALCA, the occurrence of other anti-glycan antibodies and their median titers in CD at diagnosis was also similar to those observed in Crohn's disease (celiac disease gASCA: 33.1 U/mL, AMCA 79.3 U/mL, ALCA 21.5 U/mL, ACCA 68.4 U/mL vs Crohn's disease gASCA: 48.3 U/mL, AMCA 55.5 U/mL, ALCA 25.4 U/mL, ACCA 46.2 U/mL). In addition, the positivity rate for any anti-glycan antibody was also comparable in these patient groups (CD vs Crohn's disease: 65.9% vs 59.4%, P = NS). In addition, sensitivity, speci ficity, positive and negative likelihood ratios in celiac disease are comparable to that observed in Crohn's disease. Consequently, in patients with gastrointestinal symptoms, the presence of gASCA, AMCA, or ACCA may not only suggest underlying Crohn's disease but may also be associated with untreated CD. At the same time, and based on our results, ALCA and anti-OMP proved to be specific but relatively non-sensitive markers for Crohn's disease.

Current data advocate that in both CD and Crohn's disease patients have a primary defect in intestinal permeability that is also shared by a subgroup of relatives. In CD, it is also apparent that the exposure to gluten results in mucosal inflammation and the consequent tissue damage further abrogating the primary gut barrier defect, while gluten removal significantly reduces or resolves the enhanced intestinal permeability. It is assumed that these gliadin-induced processes (intestinal barrier impairment) are the reasons for antibody generation in this disease. This assumption is further confirmed by the fact that a correlation was found between anti-glycan markers and TGA or EMA in this study and also that the antibody status fundamentally

changed following the introduction of GFD. gASCA and other anti-glycan antibodies totally disappeared from the examined coeliac patients following the strict and long-term gluten-free diet.

In the present study, we also established that the kinetics of antibody disappearance is variably sensitive to the length of GFD. Of the anti-glycan antibodies, AMCA and ACCA declined most rapidly, right after the TGA titer started to diminish.

We evaluated the possible relationship between serological response and the clinical presentation of the disease. Patients with multiple seroreactivity to glycans, more commonly presented with severe malabsorption as compared to those without any reactivity against any glycan at all (63% vs 22%, P = 0.019), and accounted for 53% of all malabsorption cases. Among the patient groups, the TGA titer reached the highest value (115.9 U/mL vs others: 60.9 U/mL, P = 0.016) in those presenting with malabsorption, further supporting enhanced intestinal permeability as a likely component involved in antibody formation. It is well known that the intestinal damage is most pronounced in the malabsorption cases and TGA is a good marker for tissue injury. We must note however, that the number of subjects in different clinical presentation groups were limited, thus further studies with a larger cohort of CD patients are needed to confirm these findings.

The latest data raise the possibility that the presence of antimicrobial antibodies may be at least partially related to genetic susceptibility. In coeliac patients, we did not observe any correlation with the presence of anti-glycan antibodies and the NOD2/CARD15 variant. An inheritable trait of anti-microbial antibody formation is unlikely in CD, since we did not find a higher prevalence of ASCA (9.1% vs 14%) and anti-OMP (12.1% vs 20%) as compared to the controls in the 66 unaffected, first-degree relatives (siblings) of this cohort.

The presence of the serological response is assumed to be a reaction due to the permanent presence of the elements of intestinal microflora which resulted from increased bacterial translocation. The significance of the enhanced bacterial translocation out of the small bowel in the anti-microbial antibody formation is further supported by the fact that the presence of the serological response among patients with Crohn’s disease is mainly characteristic for those with complicated (stricturing or penetrating) small bowel involvement and is rarely observed in the isolated colonic disease or in patients with ulcerative colitis. At the same time, the recovered gut barrier function protects against the invasion of microbes or their components leading to the cessation of anti-microbial antibody formation. In CD, this process may be justified by the observation

that the serological response is a temporary phenomenon. Antibodies disappear completely as a result of the lack of gliadin exposition and the subsequent healing of the intestinal mucosa.
Our data also call for additional basic research to explore the exact mechanism of immune responses to commensal enteric bacteria as well as the possible clinical significance of the bacterial translocation in the pathogenesis or the complications of these diseases as it is well established in other clinical conditions such as liver cirrhosis, acute pancreatitis or sepsis.

**Cirrhosis patients**

To our knowledge, this is, to date the largest study to investigate the complex association between serum levels of the lectin pathway molecules and the disease etiology along with severity in a large cohort of patients with cirrhosis. Of the lectin pathway of the complement system molecules, two sPRM (FCN-2 and FCN-3) and one effector molecule were evaluated (MASP-2).
The serum level of lectin pathway shows high variability between individuals even in healthy subjects. In this study, the median MASP-2 level (102) and MASP-2 deficiency were identical to the previously disclosed values, while FCN-2 and FCN-3 levels were lower. This difference is supposedly due to the diversity of materials and methods used for this research. As for MASP-2, we used a commercially available assay widely used in complement/lectin studies. However, we used in-house double antibody sandwich ELISA to measure FCN-2 and FCN-3 levels.
In the large group of stable cirrhosis outpatients (n=266), we found that the serum levels of all three molecules were significantly lower than the healthy control group.
Further explanations are needed to understand why the serum levels of cirrhosis patients decrease. According to one possible explanation, the synthetic capacity of hepatocytes is impaired in cirrhosis and the circulation is assumed to be blocked in the given organ, which results in reduced generation of molecules or their translocation into the peripheral blood. This assumption is confirmed by our result that the FCN-2, FCN-3 and MASP-2 levels gradually decrease in accordance with disease severity. There was a significant correlation between the laboratory characteristics of synthetic liver functions (serum albumin level and INR) and the various lectin levels which showed gradual decrease in accordance with disease severity.
In this study, the observed lectin levels were permanently stable intraindividually and remained unchanged even in the early phase of acute decompensation (AD), which makes it possible to use them as serological biomarkers also in the long run.

Furthermore, we performed a follow-up study with the aim to examine whether the low level of ficolins or MASP-2 deficiency can be regarded as risk factors in cirrhosis patients from the aspect of the development of clinically significant infections.

The *FCN-2* and *MASP2* gene promoters and their encoding regions involve numerous SNPs, which has an impact on protein concentration, but relatively few SNPs were identified on the FCN-3 gene.

It is clearly shown that genotype has a very little influence on average protein concentration. Apart from gene polymorphisms, the up/downregulation of these genes and the decrease/increase of serum proteins seem to be in correlation with various inflammatory and infectious diseases. Furthermore, it remains to be determined whether the non-genetic influence is as strong as genotype or even stronger. Due to these limiting factors, we analysed the serum levels of lectin pathway molecules instead of genotyping. If serological markers are used for long-term forecasting, it is important that the serum levels of this molecule are stable at least for a certain period of time and under certain circumstances. Accordingly, we determined the stability of FCN-2, FCN-3 and MASP-2 serum levels in a subgroup of patients. The median period between samplings could be compared to the period of time until the appearance of the first clinically significant bacterial infection. Neither of the serum levels of these molecules showed any significant change after this period has passed. There were no significant change either in the severity of disease during this observable period.

The low level of pattern recognition molecules leads to the insufficient recognition of microbes and reduced complement activation, opsonisation and inflammatory cytokine secretion, which results in increased susceptibility to infections. For this reason, it is rational to assume that the serum levels of lectin pathway molecules may correlate with the bacterial infection episodes of cirrhosis patients, but this aspect has not been studied yet. In the patient group examined by us, the cumulative probability of clinically significant bacterial infections was significantly higher in the case of subjects with reduced FCN-2 or FCN-3 level and it further intensified if the levels of both lectins were low. This correlation was not independent of disease severity. These results suggest that reduced ficolin levels are the consequence of the impairment of the liver cells' synthetic capacity in advanced cirrhosis and there is no substitute which could make up for their function. For this reason, lectin deficiencies can be considered important adaptive components of cirrhosis-related immune deficiency. However, it cannot be excluded that other soluble pattern recognition molecules which similarly activate the alternative pathway, but recognise other epitopes (MBL, M-ficolin, collectin 10 and 11) are able to ease the immunological impact of FCN deficiency to some degree.

From the prognostic point of view, our results did not convey any new information, but these new findings are significant from the pathological aspect. Soluble pattern recognition molecules (sPRMs) may be the targets of non-antibiotics-based infection prophylaxis. In advanced cirrhosis, when liver functions cannot be improved without liver transplantation, the correction of cirrhosis-associated immune dysfunction syndrome components can potentially decrease the frequency of developing infections which generally lead to death. In the case of MBL deficiency, the restoration of the MBL level improved survival and it was proved to be safe and efficient both in preclinical and early phase II studies. Performing similar studies could be of significant value in the case of ficolin deficiency.

In this study, the MASP-2 levels did not correlate with the frequency of clinically significant infections. It is undoubtedly the limiting factor of our study that no functional analysis was performed. For this reason, the possibility of correlation between MASP-2 activity and the examined clinical parameters cannot be excluded.

The serum level of lectin pathway molecules at the time of including patients did not show any correlation with infection-related mortality. Since complement system presents a dual role in disease susceptibility, one can expect that during on-going inflammatory process rather its excessive activation with subsequent tissue damage than its lack of activity might be harmful. This issue warrants further evaluation in future studies of lectin pathway in cirrhosis.

## MAIN NEW SCIENTIFIC FINDINGS

### Coeliac disease

1. In coeliac disease, the appearance of anti-glycan antibodies is frequent at the time of setting up the diagnosis before starting the treatment.

2. The prevalence of antibodies and their titer values are very similar to those observed in Crohn's disease and correlate with the clinical appearance of the disease, but not with the presence of the NOD2/CARD15 mutation.

3. The highest anti-glycan antibody prevalence and titer values can be be observed in the case of malabsorption, a disease which has the most severe clinical form.

4. As a result of a sufficiently long and proper gluten-free diet, antibodies totally disappear from the serum.

5. The appearance of anti-glycan antibodies is not more frequent in the healthy first degree relatives of coeliac patients.

6. Based on our findings, ASCA and other anti-glycan antibodies can be regarded as the supplementary markers of coeliac disease and gluten-free diet compliance.

### Cirrhosis

1. We were the first to report on the lectin complement pathway molecules in a large cirrhosis patient group in a complex way. Serum lectin levels correlate with the severity of liver disease and the prevalence of portal hypertension. Lectin levels did not correlate with the etiology of cirrhosis.

2. In this study, the observed lectin levels were permanently stable intraindividually and remained unchanged even in the early phase of acute decompensation, which makes it possible to use them as serological biomarkers also in the long run.

3. These results suggest that reduced ficolin levels are the consequence of the impairment of liver cells' synthetic capacity in advanced cirrhosis and there is no substitute which could make up for their function. For this reason, lectin deficiencies can be considered new adaptive components of cirrhosis-related immune deficiency.

4. From the prognostic point of view, our results did not convey any new information compared to the currently available clinical prognostic factors and they are not suitable for inclusion into the matrix model of risk assessment, because they cannot be used as independent risk factors in infection prognosis.

5. Lectins may be the targets of non-antibiotics-based infection prophylaxis in cirrhosis patients.

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## List of publications related to the dissertation

1. **Földi, I.**, Tornai, T., Tornai, D., Sipeki, N., Vitális, Z., Tornai, I., Dinya, T., Antal-Szalmás, P., Papp, M.: Lectin-complement pathway molecules are decreased in patients with cirrhosis and constitute the risk of bacterial infections.
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