Significance of upper endoscopy and serological markers in diagnosis of paediatric inflammatory bowel disease

Ph.D. thesis

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Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract which have been empirically defined by clinical, pathological, endoscopic, laboratory and radiological features. The etiology of IBD is currently unknown. Inflammation is hypothesized to result of inappropriate activation of mucosal immunity caused by environmental factors in genetically susceptible individuals. According to international studies published in recent years, incidence of IBD, particularly CD, is continuously rising. IBD is diagnosed in every fourth patient in their childhood or adolescence.

Recent study showed that up to 15-20% of paediatric patients and 5-15% of adult patients with colonic involvement had diagnostic difficulties whether they have UC (ulcerative colitis) or colonic CD (Crohn disease). **Upper endoscopy** may help to establish the definitive diagnosis. "ESPGHAN's Porto Working Group (2005) has recommended routine upper endoscopy at the initial evaluation of children suspected to have IBD (except for undoubtedly distal UC), although it has not been evaluated so far.

Recently, in large multicentric and population-based studies 7-8 % of adult patients with CD were reported to show macroscopic upper gastrointestinal tract involvement (extending to the terminal ileum). The majority of the European and North-American studies as well as an Australian study reported higher upper gastrointestinal involvement in paediatric patients suffering from CD although similar prevalence rate has been reported in adults, too. Routinely (regardless of symptoms) performed esophagogastroduodenoscopy (EGD) revealed macroscopic lesions in 40-64% of paediatric CD patients. Furthermore, microscopic lesions were found in more than 70% of patients with CD.

Up to now, no data have been available about the prevalence of upper gastrointestinal involvement in paediatric IBD in Hungary. Furthermore, there is no large prospective study regarding the diagnostic yield of upper endoscopy at the diagnostic procedure of IBD.

Especially in children, it is of great importance to investigate non-invasive serological markers which may have an appropriate diagnostic value. **Mannose-binding lectin (MBL)** is an important component of innate immunity. MBL is a pattern-recognition molecule that activates the lectin pathway of the complement system, irrespectively of antibodies. MBL can

directly opsonize microorganisms and enhance their uptake by phagocytic cells. MBL is primarily synthesized in the liver and it predominantly circulates as a serum protein. In addition, intestinal MBL2 gene expression has been found using a commercial cDNA library. Furthermore, MBL binds to apoptotic and necrotic cells and facilitates uptake by macrophages. Recently, a new role for MBL as a Toll-like receptor (TLR), co-receptor in directing intracellular signalling has been identified.

Low MBL level was observed in up to 40% and the prevalence of MBL deficiency is 8-10 % in the normal population. MBL deficiency has been associated with increased susceptibility and severity of infections, especially in children and in immune-compromised patients. Furthermore, MBL pathway may be involved in the development of various autoimmune diseases (e.g., IBD and coeliac disease). The role of MBL in IBD is still controversial. Until now, the prevalence of MBL deficiency has not been investigated in paediatric IBD.

The search for the underlying trigger of abnormal intestinal inflammation characteristics of IBD has led to the discovery of antibodies present specifically in the blood of patients with CD and/or UC. Several autoantibodies have been described in IBD. The two most intensively studied conservative antibodies are autoantibodies to neutrophils [perinuclear anti-neutrophil cytoplasmic antibodies (pANCA)], primarily associated with UC and anti-Saccharomyces cerevisiae antibodies (ASCA), primarily associated with CD. The clinical importance of **exocrine pancreas antibodies (PAB)** and **autoantibodies against intestinal goblet cells (GAB)** is understudied in paediatric IBD patients.

Relatively low prevalence of PAB was detected in adult patients with CD (27-39%), using indirect immunofluorescence (IIF). The determination of autoantibodies against exocrine pancreas by IIF using human cells transfected with the recently identified proteoglycans CUZD1 and GP2 as recombinant target antigens (recombinant pancreas antigen 1 and 2: rPAg1 and rPAg2) represents a new dimension in the serological diagnosis of IBD.

Previously, GAB has been described with a prevalence of 28-30% in UC and with a prevalence of 20% in first-degree relatives of IBD patients. Moreover, PAB, rPAB and GAB have not been examined in a large cohort of paediatric IBD, and data on specificity and sensitivity are contradictory in adult IBD.

Aims

Our aim was to assess the prevalence of upper gastrointestinal involvement in children with IBD and determine the real diagnostic yield of upper endoscopy In addition, we evaluated the association between endoscopic and histological lesions with disease activity indexes and laboratory parameters. Furthermore, we investigated the diagnostic value and clinical utility of different serological markers (such as MBL, PAB, rPAB, GAB, ASCA and pANCA) which may play a role in the diagnosis of paediatric IBD as well as their association with phenotype, disease activity indexes, medical therapy, extraintestinal manifestations and NOD2 genotype. The main goals of our study were as follows:

1.1. Investigation of endoscopic and histological lesions as well as their associations with disease activity indexes and laboratory parameters in IBD patients who had upper endoscopy.
1.2. Determination of the real diagnostic yield of upper endoscopy in CD patients.
1.3. Characteristics of localization, disease activity and laboratory parameters in patients with CD who underwent upper endoscopy.

2.1. Investigation of MBL levels and MBL deficiency as well as their association with other serologic markers in patients with IBD.
2.2. Characteristics of MBL levels, clinical phenotype, CRP, and actual disease activity in patients with IBD.
2.3. Investigation of MBL levels and NOD2/CARD15 genotype in CD.

3.1. Determination of diagnostic accuracy of PAB, rPAB, and GAB and evaluation of the serotype-phenotype correlation.
3.2. Determination of diagnostic accuracy of ASCA and pANCA antibodies as well as analysis of the serotype-phenotype correlation.
3.3. Association between NOD2/CARD15 genotype, serum antibodies and phenotype in CD.

Patients and Methods

1. Patients

1.1. Determination of diagnostic yield of upper endoscopy

Between 2007-2009 on the basis of HUPIR (Hungarian Pediatric IBD Registry) database 237 patients [male/female (m/f) ratio: 125/112, mean age: 13.15 years (range: 1.2-18 years)] underwent upper endoscopy, 176 of them had CD, 48 UC, and 13 IBD-unclassified (IBD-U).

1.2. Determination of MBL

One hundred and seven consecutive patients with paediatric-onset CD [male/female (m/f) ratio: 64/43, mean age: 14.1 years (range: 5.3-20 years)], 52 patients with paediatric-onset UC [m/f ratio: 22/30, mean age: 14.0 years (range: 6-19.7 years)], and 95 age-and sex-matched controls were included in this study

1.3. Determination of PAG, rPAg, GAB, ASCA és pANCA antibodies

Our study included 103 consecutive patients with pediatric-onset CD [male/ female (m/f) ratio: 63/40, median age: 13.9 years (range: 5.3-19.6 years)], 49 patients with pediatric-onset UC [m/f ratio: 22/27, median age: 12.5 years (range: 6-19.7 years)], and 104 age-and sex-matched controls.

1.4. Detection of NOD2/CARD15 mutations

NOD2/CARD15 mutations were determined in 44 consecutive patients with paediatric-onset CD [male/female (m/f) ratio: 19/25, mean age: 13.3 years (range: 5.3-18 years)].

Prospectively, blood samples were obtained for measurement of MBL, PAB, rPAB, GAB, ASCA, pANCA, complete blood count and C-reactive protein (CRP) as well as for determination of NOD2/CARD15 mutations.

2. Methods

2.1. Determination of diagnostic yield of EGD

On behalf of the Hungarian Paediatric Gastroenterology Society, prospective nationwide registry of paediatric IBD was launched on the 1st of January, 2007. Cooperation of 27 institutes (clinics, hospitals, outpatient departments) has ensured the coverage of the whole country. Questionnaires were filled in by paediatric gastroenterologists, who made the diagnosis of IBD. Newly diagnosed IBD patients younger than 18 years were registered. Data

of newly diagnosed paediatric IBD patients recorded in the period between the 1st of January 2007 and the 31st of December 2009 (36 months) were investigated. Data of the registered patients were analyzed based on the diagnosis, endoscopic, histological and imaging findings as well as on the basis of disease localization and disease activity.

2.2. MBL assay

Serum concentration of MBL was detected by means of the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) system adopted from Minchinton et al. using monoclonal mouse antihuman MBL antibody. According to the recommendations of the manufacturer, low MBL concentration was defined as a serum level <500 ng/mL and MBL deficiency as <100 ng/mL.

2.3. Antibody assays for PAB, rPAB, GAB, ASCA, and ANCA

Presence of PAB, rPAB, GAB, ASCA and ANCA was determined in an commercially available IIF assay (EUROIMMUN AG, Luebeck, Germany) according to the manufacturers' instructions.

2.4. Detection of NOD2/CARD15 mutations

The three NOD2/CARD15 variants, Arg702Trp, Gly908Arg, Leu1007fs, were typed using polymerase chain reaction/restriction fragment length polymorphism. NOD2/CARD15 variants were detected by denaturing high-performance liquid chromatography.

3. Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 5 (GraphPad, San Diego CA, USA). χ^2-test and Fisher's exact test were used to evaluate endoscopic and histologic differences, as well as differences of presence of MBL deficiency and presence of ASCA, pANCA, PAB, rPAB, and GAB antibodies between CD and UC groups, as well as within subgroups of IBD patients according to disease localization. Spearman's rank order correlation was calculated to test the association between clinical activity indexes (PCDAI, PUCAI) and laboratory parameters. Mann-Whitney-test was performed to compare the values of PCDAI, PUCAI, laboratory parameters and age differences between subgroups of IBD patients statistically. Logistic regression analysis was also performed to assess the complex associations between clinical phenotype and serology profile. A p value of <0.05 was considered as significant.

Results

1.1. Endoscopic and histological findings as well as their associations with disease activity indexes and laboratory parameters in patients with IBD

Four hundred and twenty patients with IBD were registered between 1st of January 2007 and 31th of December 2009. The number of patients with CD was remarkably higher than the number of patients with UC [265 (63%) vs. 130 (31%), p<0.0001]. Fifty-four percent of the patients with IBD were male and 46% of them were female. The mean age was 13.2 years (range:1.2-18 years). Two hundred and thirty-seven (56%) patients (112 girls, 125 boys, mean age:13.15 years, range:1.2-18) underwent upper endoscopy.

Macroscopic lesions were found in 64% of patients with CD and in 40% of patients with UC (CD vs. UC p=0.003). Histological lesions were noted in 71% of CD patients and in 48% of UC patients (CD vs. UC p=0.0035). Abnormal histological findings with normal macroscopic lesions at upper endoscopy were observed in 35 (15%) paediatric IBD patients (26 CD, 7 UC, 2 IBD-U). Three of these patients had granuloma. Nineteen percent (12/62) of all granulomas were identified in the UGI tract and 13% (8/62) of these were only there. However, only 1 (2%) patient with colitis had granuloma in the UGI tract, changing the diagnosis from IBD-U to CD.

CRP and PLC values were significantly higher [p(CRP)=0.022, p(PLC)=0.034] and Se Fe levels were significantly lower (p=0.0296) in patients with macroscopic lesions than in patients without macroscopic lesions of UGI tract in CD. However, PCDAI and Htc values were not different in patients with macroscopic lesions and in patients without macroscopic lesions of UGI tract in CD. The histological findings were not associated with PCDAI and laboratory values in CD.

1.2. Diagnostic yield of EGD

Macroscopic and/or histological lesions were noted in 141 (80%) CD patients. Erosion, ulcer, aphthous lesions, cobblestoning and the presence of L4 granuloma were found in 55 (31%) CD patients. Nevertheless, the majority (65%) of our CD patients had ileal (isolated ileal or ileo-colonic) involvement. When we evaluated these findings in the subgroup of patients with colitis (L2) without colonic granuloma, EGD helped to establish the final diagnosis in 16 (9%) CD patients. According to the above-mentioned criteria, diagnostic yield

of EGD was 9% (16/176) for CD. Macroscopic and microscopic lesions were unspecific in UC patients and did not help to establish the diagnosis.

1.3. Characteristics of localization, disease activity and laboratory parameters in patients with CD who underwent upper endoscopy

We analyzed the extent of the disease in 143 paediatric patients with CD in accordance with the Montreal criteria. UGI and ileocolonic involvement, the so-called „panenteric" phenotype (L3+L4) was found in 42% of all CD patients and in 80% of patients with UGI involvement. In younger patients (under 8 years and between 8-14 years) L3+L4 involvement was higher than in patients who were older than 14 years, although the difference was not statistically significant. There were no patients with isolated UGI involvement.

Disease activity index was available in 85 CD patients with UGI involvement. The majority of patients had moderate-severe disease activity. The mean PCDAI was 33.0 (range: 7.5-67.5) in CD. We did not find significant differences in the activity index values of CD patients with UGI involvement and patients not showing UGI involvement. Analyzing the association between localization and disease activity in CD children with L4 involvement (n=41), we established that mean PCDAI was the highest in patients with L3+L4 involvement. Nevertheless, difference between subgroups of IBD patients concerning disease localization did not reach the significance level.

PCDAI correlated positively with CRP (n=103, R: 0.46, p<0.0001), with Htc (R: 0.34, p<0.0001), with PLC (R: 0.37, p=0.0001) and with low level of Se Fe (R:-0.26, p=0.0092) in patients with CD.

2.1. MBL levels and MBL deficiency in patients with IBD and their association with other serological markers

Low MBL levels (defined as < 500 ng/mL) were found in 34 of 107 (31.8%) patients with CD, in 18 of 52 patients (34.6%) with UC, and in 13 of 95 (13.7%) controls. MBL deficiency (defined as < 100 ng/mL) was found in 10 (9.4%) patients with CD, 6 (11.5%) patients with UC and 9 controls (9.5%). We noted significantly lower median MBL levels in both CD and UC patients compared to controls (CD, p=0.04, UC, p=0.004, IBD, p=0.007). Furthermore, prevalence of low MBL level (< 500 ng/mL) was significantly higher in both CD and UC groups than in controls (CD, p=0.002, UC, p=0.006, IBD, p=0.001). Nevertheless, prevalence of MBL deficiency (< 100 ng/mL) was similar in IBD patients and in controls. No significant difference was observed in the prevalence of MBL deficiency and

low MBL level as well as in median MBL levels between UC and CD patients. Moreover, we did not find any significant association between low MBL level or MBL deficiency and ASCA/pANCA in CD or in UC.

2.2. Association between MBL levels, clinical phenotype, CRP, and actual disease activity in patients with IBD

Low MBL level (< 500 ng/mL) was associated with isolated ileal involvement (p=0.01) in patients with CD. In addition, MBL deficiency (< 100 ng/mL) was related to male gender (p=0.004) in CD. Nevertheless, MBL level or deficiency was not associated with medical therapy, need for surgery, or extraintestinal manifestations - neither in CD nor in UC. Additionally, MBL level was not associated with CRP and actual PCDAI in CD or with CRP and PUCAI in UC. Moreover, CRP values correlated positively with PCDAI in CD (n=86, R: 0.617, p<0.0001). However, CRP values were not significantly associated with PUCAI in UC.

2.3. Association between MBL levels and NOD2/CARD15 genotype in CD

Prevalence of NOD2/CARD15 genotypes was available in 44 patients. MBL deficiency was not associated with NOD2 variants. Prevalence of NOD2/CARD15 mutations was not statistically different between patients with high (> 500 ng/mL, 20.6%) and low (< 500 ng/mL, 33.3%) MBL levels.

3.1. Diagnostic accuracy of PAB, rPAB, and GAB and association with disease phenotype in CD and UC

Presence of PAB and rPAB (IgA or IgG) antibodies was significantly higher in CD (34% and 35.9%) and UC (20.4% and 24.5%) compared to controls (0% and 0%, p<0.0001). The combination of PAB and/or ASCA/pANCA improved sensitivity of serological markers both in CD (87.4%) and in UC (79.6%). Specificities were 89.3% and 93.2%, respectively. The positive predictive value (PPV) was 89.1% in CD for the combination of the markers and negative predictive value (NPV) was 87.6% (in UC, PPV, 93.2%, NPV, 82.2%).

GAB positivity was significantly higher in patients with UC compared to CD and controls, respectively (UC, 12.2%, CD, 1.9%, controls: 1.9%, p=0.02).

Presence of PAB, rPAB, and GAB was not associated to clinical presentation, medical therapy, need for surgery or extraintestinal manifestations in either CD or in UC. The PAB and rPAB positivity was numerically higher in patients with colonic (28.6% and 27%) and

ileocolonic (60% and 59.4%) CD than in ileal disease (11.4% and 13.5%). However the difference was not statistically significant.

3.2. Diagnostic accuracy of ASCA and pANCA and association with disease phenotype in CD and in UC

Of the 103 CD patients studied, 72.8% were ASCA positive (either IgA or IgG). Presence of ASCA (either IgA or IgG) was significantly higher in CD (72.8%) compared to UC (26.5%), and control (4.8%) patients.

ASCA positivity was associated with complicated disease behavior (p=0.0003) [stenosing (p=0.02) and/or penetrating disease behavior (p=0.0003)] and perianal complications (p=0.01) in CD. No association was found with the location in patients with CD. The frequency of stenosing and penetrating disease, and ileocolonic involvement increased with increasing number of immune responses, whereas inflammatory behavior (B1) showed inverse correlation with the number of ASCA. There was no significant difference with regard to the need for surgery between ASCA positive and ASCA negative patients (need for surgery: 12% vs. 3.6%). ASCA was not associated with medical therapy and extraintestinal manifestations.

ASCA positivity was found in 13 UC patients (26.5%), however 7 of these patients had progressive sclerosing cholangitis (PSC).

PANCA were detected in 38 (77.5%) of 49 UC and in 34 (33%) of 103 CD patients**.** Presence of pANCA was not associated with clinical presentation, medical therapy, need for surgery or extraintestinal manifestations - neither in CD nor in UC.

3.3. NOD2/CARD15 genotype, serum antibodies and phenotype in CD

NOD2/CARD15 genotypes were known for 43 CD patients. Mutations of NOD2/CARD15 were detected in 13 (30.2%) CD patients. There was no association of ASCA, PAB, rPAB and pANCA antibody status to NOD2/CARD 15 genotype. Three NOD2 carriers were PAB and ASCA negative, and two NOD2 carriers were PAB, ASCA and pANCA negative. NOD2 mutations were not related to age at onset, disease location and disease behavior. Nevertheless, NOD2 variants were significantly related to steroid refractory disease and administration of infliximab.

Conclusions

1. According to our study, frequent upper gastrointestinal involvement can be detected in paediatric patients with CD and UC, but abnormalities in UC are unspecific. Upper endoscopy (ulcer, erosion, aphthous lesions and granuloma) help to establish the final diagnosis in one third of patients with CD. There is a further decrease in this ratio if CD patients with terminal ileal involvement and with granuloma in the colon are not taken into consideration since in these cases upper endoscopy does not provide a diagnostic help. EGD plays a crucial role in every tenth child with CD in diagnosis (diagnostic yield). In these cases with isolated colitis, serpiginous ulcer and aphthous lesions on EGD as well as histologically identified granulomas in the upper gastrointestinal tract may establish a definitive diagnosis of CD. For this reason, in the diagnostic procedure in paediatric patients with IBD upper endoscopy is recommended.

2. In nearly 50% of children with CD, upper gastrointestinal and ileocolonic involvement, the so-called „panenteric" phenotype (L3+L4) has been found. This finding suggests that extensive disease localization is common in paediatric CD which can influence the choice of the treatment strategy and the prognosis.

3. In patients with CD showing upper gastrointestinal macroscopic lesions, CRP and thrombocyte values are significantly higher, serum iron values are significantly lower than in patients without macroscopic lesions. According to our findings, there is a close association between laboratory values and endoscopic findings in CD. In case of the above-mentioned difference in laboratory values, it is more likely that there will be signs of macroscopic pathology in the upper gastrointestinal tract in children with CD.

4. The MBL serum concentrations are significantly lower and the ratio of patients with low MBL level (< 500 ng/ml) is higher in paediatric patients with IBD than in controls – both in CD and UC. In CD, prevalence of low MBL level (< 500 ng/ml) is significantly higher in isolated terminal ileal involvement compared to other localizations. Our results suggest that low MBL level is associated with paediatric IBD and terminal ileal involvement in CD. Furthermore, it may be considered an additional factor in the pathogenesis of paediatric IBD.

5. Although PAB, rPAB és GAB antibodies are specific for IBD but the sensitivity is limited as well as there are lack of correlation with clinical phenotype. Low sensitivity suggests that these markers can not be used separately in diagnose of IBD. Combined

application of antibodies (PAB and/or ASCA/pANCA) improves sensitivity which enables that these antibodies can be applied in everyday practice.

6. In our study we observed association between ASCA positivity and complicated (stenosing and penetrating) behaviour as well as perianal disease in CD. Above-mentioned data confirm that ASCA positivity may be a marker for more aggressive disease course in CD.

7. Presence of NOD2/CARD15 variants was significantly associated with the need for more intensive therapy (steroid refractory disease and infliximab use) in CD. Based on these data, NOD2 variants may play a role in predicting clinical response to treatment.

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Publications

Publications related to the thesis

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