Serological markers in diagnosis of pediatric inflammatory bowel disease and as predictors for early tumor necrosis factor blocker therapy

Short title: Serological markers in pediatric IBD

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ABSTRACT

Objective: To describe the prevalence of serological markers in newly diagnosed treatment naïve pediatric inflammatory bowel disease (IBD), their utility in differentiating Crohn's disease (CD), ulcerative colitis (UC) and symptomatic non-IBD patients and whether serological markers are associated with early TNF blocker treatment.

Material and methods: Ninety-six children and adolescents < 18 years, 58 with IBD and 38 symptomatic non-IBD controls were included. At diagnosis and after 1-2 years, serological antibodies (anti–Saccharomyces cerevisiae antibodies (ASCA), perinuclear anti-neutrophil cytoplasmic antibody (pANCA), flagellin expressed by Clostridial phylum (anti-CBir), outer membrane porin of Escherichia coli (anti-OmpC), Pseudomonas fluorescens associated sequence (anti-I2)), CRP, ESR, and fecal calprotectin were analyzed. The choice of treatment was made at the discretion of the treating pediatrician.

Results: Of the IBD patients 20 (36%) and 26 (47%) were positive for ASCA and pANCA compared to 3(8%), $p<0.01$ and 10 (27%), $p=0.04$ of the controls. Thirteen (72%) of UC patients were pANCA positive, versus 13 (35%) of CD patients ($p<0.01$). None of the UC patients was ASCA positive versus 20 (54%) of CD patients ($p<0.0001$). Compared to conventionally treated patients, the 18 (49%) TNF blocker treated CD patients had higher presence of ASCA ($p<0.01$) lower presence of pANCA ($p=0.02$) and higher levels of fecal calprotectin, CRP and ESR at diagnosis. In multivariate analyses ASCA and pANCA status, but not CRP, ESR or calprotectin, were independently associated with early TNF blocker treatment.

Conclusions: ASCA and pANCA status were associated with having IBD and with early TNF blocker treatment in CD.

Key Words: IBD, pediatric, serological markers, ASCA, pANCA**,** biologic therapy, biomarkers, TNF blocker, Crohn´s disease, ulcerative colitis

INTRODUCTION

The inflammatory bowel diseases (IBD) Crohn's disease (CD) and ulcerative colitis (UC) are chronic, lifelong diseases in the gastrointestinal tract with frequent extra-intestinal manifestations and severe complications. The incidence of IBD is rising and up to 25% of cases are diagnosed in childhood and adolescence (1, 2). Pediatric IBD is often characterized by an extensive disease distribution and an aggressive disease course (3, 4). It is considered important to initiate appropriate treatment early to avoid complications. Tumor necrosis factor (TNF) blockers represent a potent treatment option but are hampered with potentially serious side effects, high cost and are not needed by all patients. Prognostic markers to select children and adolescents for early aggressive treatment are needed.

Among autoantibodies and antibodies against microbial antigens in CD and UC patients, perinuclear anti-neutrophil cytoplasmic antibody (pANCA) and anti–Saccharomyces cerevisiae antibodies (ASCA) are the most studied (5). Antibodies against the outer membrane porin of Escherichia coli (anti-OmpC), antibodies against a Pseudomonas fluorescens associated sequence (anti-I2), and flagellin expressed by Clostridial phylum (anti-CBir1), have more recently been investigated in IBD patients (5, 6). Studies in IBD patients have indicated that these serological markers can predict aggressive CD behavior and help to diagnose and guide therapy (7, 8). The significance of serological markers to predict disease course and their stability over time is still not fully explored in pediatric IBD patients.

Our aims were to describe the prevalence of serological markers in treatment naïve pediatric IBD patients at the time of diagnosis and their utility in differentiating CD, UC and symptomatic non-IBD patients. We also wanted to assess whether the presence of autoantibodies was associated with the initiation of early TNF blocker therapy. Moreover, we wanted to compare the clinical relevance of serological markers to traditional markers of inflammation, such as C- reactive protein (CRP), elevated sedimentation rate (ESR) and

fecal calprotectin.

MATERIALS AND METHODS

Patients and controls

A total of 96 children up to 18 years of age were included in the present study, which was part of a large-scale prospective study of IBD in South-Eastern Norway (the IBSEN II- study) (1). Fifty-eight treatment- naïve pediatric patients with newly diagnosed IBD were included, 39 with CD, 18 with UC and one with inflammatory bowel disease unclassified (IBDU). These patients were recruited from the catchment areas of two university hospitals in Norway, Akershus University Hospital and Oslo University Hospital, and comprised all IBD patients diagnosed in the inclusion period from May 1st 2005 to December 31st 2007. A prescheduled follow-up examination was performed within two years of diagnosis (9). Thirty-eight children and adolescents, who were referred to the two hospitals during the inclusion period and initially believed to have IBD based on symptoms (most commonly abdominal pain and altered bowel habits) and laboratory findings, were included as a non-IBD control group. Of the 38 control patients, two were diagnosed with celiac disease, one of which also had a juvenile polyp, and one patient had hypogammaglobulinemia. The remaining 35 patients displayed no evidence of inflammation during work up indicating functional gastrointestinal disorders. According to medical records none of the non-IBD symptomatic patients have developed IBD as of September 2016, which gives a follow-up of 7-12 years. At inclusion and at follow up, the same protocols with registrations, examinations and laboratory tests were followed both in the IBD patients and in the non- IBD control group. The Vienna, and later the Paris classification, was used to characterize the disease distribution and behavior (10, 11).

Clinical, endoscopic, radiological and laboratory data

Age, gender, symptom duration, disease and family history of the IBD cohort were registered as previously described (1). Symptoms were scored according to the pediatric Crohn´s disease activity index (PCDAI) (12) and the pediatric ulcerative colitis activity index (PUCAI) (13). Weight, height, and pubertal development were recorded. The IBD diagnosis was made in accordance with the Porto criteria (14), and all patients and non-IBD controls were examined with upper and lower endoscopies and with magnetic resonance imaging (MRI) studies to examine the small bowel. Fecal samples were tested for fecal calprotectin (Bühlmann, Basel, Switzerland) and pathogenic gut bacteria. Blood specimens were collected for analyses including ESR, CRP, hematocrit, complete blood count, and albumin level. Serum samples from the IBD patients and controls were stored at minus 70 degrees Celsius until analysis for serological markers. Details regarding CRP, ESR and calprotectin levels are previously published (1, 9).

Analyses of serological markers

Serum samples were analyzed for the presence and titers of antibodies against ASCA IgA, ASCA IgG, OmpC, CBir1, and I2, and for the presence of pANCA. Standardized enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay were used. All analyses were performed by Prometheus laboratories (San Diego, CA, USA). The laboratory was blinded for IBD or non-IBD diagnosis. Antibody levels were expressed by ELISA units (EU/ml), except for pANCA, which was classified as detected or not detected. Reference values (EU/ml, ELISA) for antibodies were: ASCA IgA<8.5, ASCA IgG<17.8, OmpC IgA< 10.9, CBir1 IgG< 78.4 and I2< 368, which were relative to a Prometheus Laboratory Standard, derived from a pool of patient sera with well-characterized disease found to have reactivity towards these antigens (6). Titers above the reference value were

considered a positive serologic response. All serologic markers from baseline and follow-up were analyzed at the same time point.

Treatment

Treatments and disease course, from diagnosis until scheduled follow-up, were recorded. Initial treatment options were 1) exclusive enteral nutrition (EEN) in CD patients, or 2) corticosteroids and/or 5-aminosalicylic acids (5-ASAs) in CD and UC patients. Patients receiving EEN avoided all other types of food and liquids except the chosen formula and water for 6-8 weeks. Corticosteroids were given as prednisolone 1-2 mg/kg or budesonide capsules (9 or 6 mg initial dosage depending on weight) and tapered slowly.
As maintenance therapy azathioprine was in general started simultaneously with EEN or corticosteroids and/or 5-ASA. The indication for treatment with TNF blockers (infliximab/adalimumab) was failure to enter remission with the treatments mentioned above, or early relapse (within weeks after primary induction). Exclusive enteral nutrition (EEN), 5-aminosalicylic acids (5-ASAs), corticosteroids, azathioprine and methotrexate were considered conventional treatments. Biologic therapy with TNF blockers was regarded as aggressive treatment. Treatment was decided individually at the discretion of the treating pediatrician.

Statistical analyses

Data were described with median and observed range (continuous variables) and with frequencies and percentages (categorical variables). Due to a limited sample size, non-parametric methods were used. The Mann-Whitney Wilcoxon test for unpaired samples was used for comparisons between groups with regards to continuous variables. When comparing

measurements at baseline and after treatment, Wilcoxon signed rank test was used. Crude associations between pairs of categorical variables were assessed with Chi-square test. Univariate and multiple logistic regression models were fitted to estimate odds ratios (OR). The results were also expressed as probabilities. All tests were two-sided. P-values <0.05 were considered statistically significant. We regarded our study as an exploratory analysis; therefore we did not adjust for multiple testing. All analyses were performed using SPSS, statistical software SPSS version 22 (SPSS Inc., Chicago, Il, USA) and Stata version 9.

Ethical considerations

The study was performed in accordance with the Helsinki declaration and with approval of the regional committee for medical research ethics. Written informed consent was collected from all parents.

RESULTS

In total, 55 patients with IBD (37 with CD and 18 with UC) and 37 non-IBD controls were eligible for analyses of the serological markers ASCA IgA and IgG, CBir, OmpC, I2, and pANCA status at diagnosis. Serology data were missing from two of the CD patients and from the IBDU patient at baseline due to technical problems. Median duration of follow-up was 20 months (range 12-24). At follow-up 31 (84%) CD patients, 14 (78%) UC patients and 5 (14%) non-IBD controls had repeated serology. All patients and controls had laboratory measurements of ESR, CRP and fecal calprotectin from baseline and follow-up. Disease distribution and treatment of CD patients are given in table 1. The three groups CD, UC and non-IBD were similar in age, but there were more males among the IBD patients compared to the control group (Table 2).

IBD versus non-IBD controls

At diagnosis, antibodies against pANCA, ASCA IgA, ASCA IgG, and I2 were significantly increased in IBD patients compared to non-IBD controls (Table 2). Antibodies against ASCA and pANCA showed the highest ability to differentiate between patients groups, with 36% and 47% of IBD patients being positive for ASCA and pANCA compared to 0 % and 27% of the non-IBD controls. Being ASCA positive gave 6 times higher odds of having IBD and a probability of 87% (OR = 6.13 95%CI (1.67-22.47), $p<0.01$ while pANCA positivity gave a probability of 72% with OR 2.6 95%CI (1.05-6.42), $p= 0.04$. The prevalence of a serological response towards I2 was also higher in IBD patients, with 38% versus 11% in the non-IBD controls, and being I2 positive gave an 84% probability of having IBD, with OR 5.1 95%CI (1.6-16.4), $p<0.01$. There were too few patients and controls with positive serological responses towards OmpC and CBir to conduct meaningful analyses (Table 2). The inflammatory markers CRP, ESR and fecal calprotectin were significantly higher in the IBD

patients compared to non-IBD controls as previously published (1) (Table 2).

UC versus CD

More than two thirds (72%) of UC patients and one third (35%) of CD patients were pANCA positive (Table 1). Moreover, patients who were pANCA positive were almost 6 times more likely to have UC compared to CD, with a probability of 85%, OR 5.8 95%CI (1.55-21.33), $p<0.01$. Being positive for ASCA IgA gave a 94% probability of having CD, as significantly more CD than UC patients had antibodies against ASCA (Table 2); none of the UC patients had positive ASCA titers. There were no statistically significant differences between CD and UC patients regarding prevalence of antibodies against I2, OmpC or CBir. CD patients had significantly higher values of CRP and fecal calprotectin compared to UC patients, whereas there were no significant differences in ESR values between the two patient groups (1) (Table 2).

Factors associated with disease behavior

Half of the CD patients (n=18) received early aggressive TNF blocker treatment. Median time to TNF blocker initiation was 2 months, (range 0.5- 6 months). They had all received azathioprine from time of diagnosis (9). These patients had a significantly higher presence of antibodies against ASCA IgA and IgG as well as higher titers of ASCA IgG compared to CD patients who received conventional treatment (Table 2). Of the conventional treated CD patients, nine (47%) received corticosteroids and/or azathioprine and six (32%) were given either EEN or 5-ASAs. If ASCA antibodies were present at baseline, CD patients had almost 9 times higher odds of receiving early TNF blocker treatment compared to being ASCA negative, with a probability of 70%, OR 8.8 95%CI (2.0-37.7), $p<0.01$.

The presence of pANCA antibodies was significantly less frequent at diagnosis in CD patients treated with TNF blockers compared to conventionally treated patients, 3 (17%) and 10 (53%)

respectively. PANCA negative patients had more than five times higher odds of receiving aggressive therapy compared to pANCA positive patients (OR=5.2 95%CI (1.11-24.13), p=0.02). All patients who received early TNF blocker treatment were either ASCA positive and/ or pANCA negative.

CD patients who were treated with TNF blockers had significantly higher levels of fecal calprotectin, CRP and ESR at diagnosis compared to conventionally treated CD patients (Table 2). In multivariate analyses, pANCA and ASCA status was associated with early TNF blocker initiation whereas CRP, ESR and fecal calprotectin failed to reach the level of statistical significance.

The treatment responses were good and similar in both conventional and TNF blocker treated CD patients, with 83% of patients in clinical remission after induction treatment. The response was sustained at two years follow-up (9).

Of the 18 UC patients, none received early TNF blocker treatment.

Change in titers over time

After treatment, there was no difference in antibody prevalence for ASCA IgA, ASCA IgG, I2, OmpC or CBir in the CD and UC patients, regardless of treatment modality. There was a trend towards higher ASCA IgA titers with time in CD patients treated with TNF blockers (p=0.058). No UC patients became positive over time for ASCA IgA or IgG. After treatment, significantly fewer UC patients tested positive for pANCA compared to at baseline, 9 (64%) vs. 13 (72%), p= 0.013.

DISCUSSION

In the present population based pediatric cohort covering all newly diagnosed children and adolescents with IBD in our catchment area, we found a significantly higher prevalence of antibodies against ASCA, pANCA, and I2 in IBD patients than in non-IBD controls. However, due to overlap between IBD and non-IBD patients in antibody prevalence, presences of these serologic markers were only associated with and not diagnostic for IBD or IBD subgroups. ASCA IgA and IgG seem, however, to be indicators of having Crohn's disease.

ASCA status was useful for differentiating between UC and CD patients and was associated with early TNF blocker therapy in our CD patients. ASCA IgA or IgG positive patients and patients with high ASCA IgG titers had a higher probability of receiving aggressive therapy with TNF blockers than those who were ASCA negative. Conversely, CD patients with pANCA autoantibodies were less likely to receive TNF blocker treatment. Consequently, being pANCA negative and/ or ASCA positive seems to be an indicator of a CD phenotype that warrants early aggressive therapy. These findings are supported by studies in adults, children and adolescents, showing that ASCA positivity is associated with an aggressive disease course with stricturing and penetrating disease behavior, small bowel involvement, and the need for early surgery (8, 15-20).

ASCA has been reported to be relatively stable regardless of disease course, medical or surgical treatment, and is therefore not suited for monitoring disease activity and treatment responses (6, 21-25). Our study confirms the stability of ASCA IgA and IgG, as the titers of both markers were stable, regardless of medical treatment modality. Antibodies towards ASCA and pANCA may be present years before the diagnosis of IBD (26, 27). Due to the stability of ASCA antibodies, ASCA status can serve as a prognostic marker even when tested later in the disease course. We observed a non-significant trend towards higher ASCA IgG

levels with time in the TNF blocker treated patients. This increase might be an age related phenomenon and not an indicator of increasing disease severity as it has been found that serologic responses towards ASCA increase with age in children with CD (28). The prevalence of ASCA positivity in our CD patients is similar to what has been reported previously (29).

PANCA has been found to be associated with distinct phenotypes in IBD. In adult and pediatric populations, pANCA positive CD patients have been reported to have an UC-like phenotype and a more benign disease course (15, 17, 28, 30), which is in line with our results. In adult UC patients, the presence of pANCA is associated with an aggressive disease course, a higher relapse rate, resistance to therapy as well as more frequent need for early surgery (31-33). The majority of our UC patients, 13 out of 18, was pANCA positive and had extensive colitis in accordance with a pediatric phenotype, with pancolitis being the most common presentation at diagnosis in UC (34). None of the UC patients received early TNF blocker treatment. This could be due to TNF blocker therapy not being standard of care in pediatric UC patients during the inclusion period of 2005-2007.

Most pediatric and adult studies have shown a 50-70% prevalence of pANCA positivity at diagnosis in UC (29, 35-37). Some studies in adult UC patients reported a lower prevalence of only 20-30 % (18, 31). In these studies pANCA status was determined 10 years after diagnosis, and this may have contributed to the low prevalence, as the stability of pANCA in treated patients has been questioned (20, 38). PANCA prevalence has been reported to be low in UC patients in longstanding remission (39). In some studies pANCA status changed after therapy and colectomy whereas others failed to show an association between disease activity and presence of pANCA (23, 29, 40). In the present study, a decline in pANCA titers was demonstrated after treatment, in both CD and UC patients. This difference was only statistically significant in the UC patients. Nevertheless, as some of our patients became

pANCA negative after therapy, pANCA status should probably be determined early in the disease course in order to be a reliable prognostic factor.

I2 was a weak indicator of having IBD, with a prevalence of around 40% in our IBD patients, compared to 11 % in the non-IBD controls. This is in accordance with previous reports in pediatric patients (41). I2 was just as frequent in UC and CD patients and was not associated with initiation of early TNF blocker treatment in our CD patients.

The prevalence of CBir in our sample was lower than what has been found in other pediatric studies, where the prevalence of CBir was reported as high as 66% in CD children less than eight years (28). The prevalence of Anti-OmpC was also low in our patients. With the low sensitivity of I2 and low prevalence of OmpC and CBir, their usefulness from a clinical perspective seems limited.

In a previous study we evaluated clinical and biochemical markers (PCDAI, disease extent, hemoglobin, hematocrit, albumin, liver enzymes, platelet count, leukocytes, ESR, CRP and fecal calprotectin) and found that ESR, CRP and fecal calprotectin were the only markers that were significantly higher in pediatric CD patients who needed TNF blocker treatment in order to gain remission compared to conventionally treated CD patients (9). In the present study we compared the predictive value of the established biomarkers CRP, ESR and fecal calprotectin with serological markers in our CD patients. Being ASCA positive and/or pANCA negative was associated with early aggressive treatment initiation in our patients. In multivariate analyses we found pANCA and/or ASCA status but not CRP, ESR and fecal calprotectin levels to be associated with early TNF blocker therapy.

Our patients comprise an unselected cohort of all newly diagnosed children and adolescents with IBD in our catchment area in the study period. This enabled us to derive a realistic estimate of the presence of serological markers in IBD patients. The use of a detailed, standardized protocol with clinical characterization, endoscopy, laboratory and radiological

work-up applied at baseline and at follow up within 2 years, gave a high diagnostic precision. Further, the choice of therapy was led by the disease severity and reflects everyday practice. The major limitation of our study is its limited sample size reducing the power to draw conclusions and reducing our ability to detect potential differences of the microbial antigens CBir, OmpC and I2 as statistically significant.

CONCLUSIONS

The prevalence of antibodies against ASCA, pANCA, and I2 were significantly higher in an unselected Norwegian cohort of pediatric IBD patients compared to a group of symptomatic non-IBD controls. ASCA and pANCA status was useful in differentiating UC and CD patients and positive ASCA and/or negative pANCA was associated with early initiation of TNF blocker therapy in CD patients. ASCA serology was stable, regardless of treatment modality, and might be a prognostic tool at any time in the disease course. Knowing whether a pediatric CD patient is ASCA positive and/or pANCA negative may be helpful for the choice of appropriate treatment early in the disease course in order to prevent restricted growth, delayed puberty and complications.

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