

Rediscovery of the anti-pancreatic antibodies and evaluation of their prognostic value in a prospective clinical cohort of Crohn's patients: the importance of specific target antigens (GP2 and CUZD1).

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List of abbreviations: ASCA: anti-*Saccharomyces cerevisiae* antibody, BT: bacterial translocation, CD: Crohn's disease, CRP: C-reactive protein, CUZD1: CUB and zona pellucida-like domains 1, ECCO: European Crohn's and Colitis Organisation, EIM: extraintestinal manifestations, ELISA: enzyme-linked immunosorbent assay, HBI: Harvey-Bradshaw Index, HR: hazards ratio, HRP: horseradish peroxidase, IBD: inflammatory bowel diseases, Ig: immunoglobulin, IIFT: indirect immunofluorescence test, IQR: inter quartile range, LPS: lipopolysaccharide, PAb: pancreatic autoantibody, PSC: primary sclerosing cholangitis, UC: ulcerative colitis, 95%CI: 95% confidence interval, TNF: tumor necrosis factor

ABSTRACT

Backgrounds & Aims: Glycoprotein 2(GP2) and CUB zona pellucida-like domain 1(CUZD1) belong to protein families involved in gut innate immunity processes and have recently been identified as specific targets of anti-pancreatic autoantibodies(PAbs) in Crohn's disease(CD). We aimed to determine the prognostic potential of novel target specific PAbs regarding long-term disease course of an adult CD patient cohort. **Methods:** Sera of 458 consecutive well-characterized IBD patients from a single referral IBD center were tested by enzyme-linked immunosorbent assay(ELISA) with isoform 4 of recombinant GP2(anti-MZGP2 and anti-GP2 IgA/IgG) and indirect immunofluorescence test(IIFT) system with GP2 and CUZD1 expressing transfected HEK 293 cells(anti-rPAg2 and rPAg1 IgA/IgG) . Clinical data were available on evolvement of complicated disease or surgical interventions as well as disease activity and medical treatment during the prospective follow-up (median, 108 months). **Results:** 12.4% and 20.8% of CD patients were positive for IgA/IgG type of anti-GP2 and anti-CUZD1, respectively, with a significant difference compared to UC ($p < 0.01$). Antibody status was stable over time. Agreement among three different anti-GP2 assays was good. Positivity for PAbs, mainly IgA subtypes, predicted a faster progression towards complicated disease course. In Kaplan-Meier analysis, time to surgery or development of perianal disease was associated with anti-GP2 IgA ($p \text{LogRank} < 0.01$) or anti-CUZD1 IgA ($p \text{LogRank} < 0.001$) positivity, respectively. Anti-CUZD1 IgA remained an independent predictor in multivariate Cox-regression model (HR:3.43, 95%CI: 1.68–7.02, $p < 0.001$). **Conclusions:** The present study has shown that specific PAbs (especially IgA subtype) are predicting complicated disease course including the development of perianal disease in CD.

Key words: serologic antibodies, anti-pancreatic antibodies, glycoprotein CUZD1, glycoprotein 2, immunoglobulin A, Crohn's disease, ulcerative colitis, disease progression

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract. Both Crohn's disease (CD) and ulcerative colitis (UC) are heterogeneous in their presentation and disease course. Serological antibodies have been reported to assist in the diagnosis, differential diagnosis, and prediction of either disease course or response to therapy in IBD¹. There is accumulating evidence from cross-sectional, longitudinal studies and meta-analyses that support the value of serological markers in identifying patients with complicated disease phenotype and increased risk of surgery in patients with CD. Anti-*Saccharomyces cerevisiae* antibody (ASCA) has been identified as the most accurate single marker in CD, yet it is questionable if other markers can identify a specific subset of CD patients².

Enhanced formation of autoantibodies against acinar cells of the exocrine pancreas in patients with CD and celiac disease was reported³. Presence of pancreatic autoantibodies (PAb) was identified first by indirect immunofluorescence technique (IIFT)⁴ and human and primate pancreas substrate has been used as the primary diagnostic test for PAb so far. PAb are present up to 40% of the patients with CD and can be used to differentiate CD from UC². Furthermore, some studies, including the one with the largest patient cohort, have suggested that presence of PAb is associated with specific disease phenotypes and their detection may be of clinical significance^{5, 6}.

More recently, the target antigens of PAb have been identified: the pancreatic major glycoprotein GP2 of the zymogen granule membrane and CUZD1 protein (CUB and zona pellucida-like domains 1)^{7, 8}. In the study of *Komorowski et al.*, they verified that the formerly reported reticulogranular pattern in tissue sections of human pancreas by IIFT is strictly correlated with the presence of anti-CUZD1, whereas droplet pattern is to the presence of anti-GP2 antibodies⁹. Furthermore, investigation

of GP2 and CUZD1 revealed that they belong to protein families involved in innate and adaptive immunity^{10, 11}. Presumably GP2 and CUZD1 may be involved in maintaining the balance between tolerance to commensal bacteria and immune response against pathogens¹².

Identification of the autoantigens has facilitated the development of new diagnostic tools for evaluation of PABs, such as enzyme-linked immunosorbent assay (ELISA) with isoform 4 of recombinant GP2¹³ or advanced CUZD1 and GP2 expressing cell-based IIFT¹⁴. Experience with these newly available techniques for PAB detection is scarce, especially on the clinical importance of PABs in CD. Recombinant GP2-based ELISA systems were used primarily in these studies. In contrast, information about anti-CUZD1 is very limited⁹. Presence of anti-GP2 was associated to stricturing behavior with perianal disease in some¹⁵⁻¹⁷ but not all studies¹⁸. However, studies were limited by cross-sectional design. Longitudinal follow-up studies are needed to unravel the clinical importance of these antibodies in the prediction of CD prognosis. For this, studies should also investigate long-term stability of PABs

The primary aim of the present study was to determine the predictive potential of the different PABs with regards to the development of disease specific complications or need for surgery in a large prospective referral CD cohort. The secondary aims were to investigate (1) the long-term stability of the PABs response (2) association of PABs formation to the clinical, serologic and genetic characteristics of CD and lastly (3) to define the agreement between the new diagnostic tools for the evaluation of different target specific PABs.

MATERIAL AND METHODS

Patient population

We performed a cohort study among adult CD and UC patients in one tertiary IBD referral center of Hungary (Department of Gastroenterology, Institute of Internal Medicine, University of Debrecen). In all, 458 well-characterized, unrelated, consecutive IBD patients with a complete clinical follow-up (CD: 271 [male/female: 120/140, median age at presentation: 25 years (inter quartile range [IQR], 19-33)] and UC: 187 [male/female: 86/101, median age at presentation, 33 years (IQR, 23-43)]) seen at our outpatient clinic were included between January 1, 2005 and June 1, 2010.

The clinical characteristics of the patients at diagnosis are presented in **Table 1**. Diagnosis of IBD was based on the Lennard–Jones criteria¹⁹. The disease phenotype (age at onset, duration, location, and behaviour) was determined according to the Montreal Classification²⁰. Blood samples and detailed clinical phenotypes were captured at inclusion. Clinical data were determined by thorough review of patients' medical records, which had been collected in a uniform format. Medical records that documented the disease phenotype, presence of extraintestinal manifestations (EIM) (for example, arthritis: peripheral and axial; ocular manifestations: conjunctivitis, uveitis, iridocyclitis; skin lesions: erythema nodosum, pyoderma gangrenosum; and hepatic manifestations: primary sclerosing cholangitis [PSC]), frequency of flare-ups (frequent flare-up: >1 clinical relapse/year)²¹, medication use (e.g., steroid, immunosuppressive and/or biological use at any time), need for surgery (resection in CD and colectomy in UC), the presence of familial IBD, smoking habits, and perianal involvement were retrospectively analyzed for the period prior to the prospective follow-up. At enrolment, clinical disease activity was calculated according to the Harvey–Bradshaw Index (HBI)²² in CD and the partial Mayo score in UC²³. In this

study we followed the ECCO (European Crohn's and Colitis Organisation) guidelines²⁴ and defined HBI ≤ 4 as a state of remission and ≥ 5 as a state of active disease. In case of UC ≤ 3 was defined as a state of remission and >4 as a state of active disease. Endoscopic activity was determined according to the Simple Endoscopic Score for Crohn's Disease (SES-CD) in CD²⁵ and the endoscopic component of the Mayo score in UC²⁶. SES-CD defines endoscopic activity ≥ 3 points and inactive disease ≤ 2 in CD, meanwhile in UC state of active disease was defined as invasive partial Mayo score ≥ 1 .

Phenotypical characterization of CD patients during prospective follow-up

CD patients were enrolled into a prospective follow-up study, where the treating IBD physicians registered laboratory data, endoscopic and imaging findings, disease activity, medical treatment, date and type of complications and surgery during regular and extraordinary outpatient follow-up visits and inpatient stays. In Hungary, a follow-up visit is usually scheduled for every 6 months at a specialized gastroenterology center (the actual interval varies between 3–6 months). The treatment algorithms, both the medical and surgical, are harmonized and followed the actual ECCO guidelines^{24, 27-30}. A need for surgery and timing of the resection is a consistent multidisciplinary decision with the collaboration of the gastroenterologist, radiologist and surgeon. Collected data were transferred and stored in a database for analysis. In October 1, 2013, all patients' charts and database were reviewed and updated for the data points mentioned above. Follow-up for a particular patient was terminated if there was no further record available. Median follow-up from diagnosis was 108 months (IQR, 65-178). In CD, complicated disease behavior was defined as the occurrence of stenosis or internal penetration. Perianal fistulizing disease was

distinguished from internal penetrating disease and evaluated separately. Need for surgery was defined as CD-related abdominal surgery (resection). **Figure 1** summarizes flow chart of the patients with CD in the cohort study.

The control group consisted of 100 age- and gender-matched healthy blood donors (male/female: 46/54, median age at presentation, 30 years (IQR,21-40)]. The control subjects did not have any gastrointestinal and/or liver disease and were selected from consecutive blood donors in Debrecen.

Serological Analysis

Blood samples were obtained at enrollment from each patient and were frozen at -80°C until testing. All the serological assays were performed in a blinded fashion without prior knowledge of the patients' diagnosis or other clinical information.

Detection of antibodies to GP2 by enzyme-linked immunosorbent assays(ELISA)

Glycoprotein 2 autoantibodies were detected in sera of patients and controls using two different ELISAs employing recombinant human GP2 isoform 4 as solid-phase antigen(anti-MZGP2 IgA and IgG QUANTA Lite® ELISA [Inova Diagnostics, San Diego, CA, Research Use Only] and anti-GP2 IgA and IgG ELISA [GA Generic Assays, Dahlewitz/Berlin, Germany]) according to the manufacturer instructions. Briefly, 100 µl of pre-diluted sera (1:100) was added to separate wells of MZGP2 or GP2 antigen-coated polystyrene microwells and incubated for 30 min. and 60 min., respectively at room temperature. Unbounded sample was then washed away and

peroxidase-conjugated goat anti-human IgA antibody or anti-human IgG antibody was added to each well and developed with ready-to-use hydrogen peroxide/tetramethylbenzidine chromogenic substrate. The reaction was terminated with sulphuric acid and optical density of the samples was read at wavelength of 450/620 nm. Results expressed in arbitrary units (AU/ml), were calculated in reference to a kit provided calibrator. Serum samples showing ≥ 25 AU/ml for anti-MZGP2 and ≥ 20 AU/ml for anti-GP2, respectively were interpreted as positive.

Detection of antibodies to GP2 and CUZD1 by indirect immunofluorescence tests (IIFT)

Anti-GP2 and anti-CUZD1 IgA and IgG were detected in sera of patients and controls using cell-based IIFT (Morbus-Crohn Mosaic 1, Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany). Biochips coated with transfected HEK293 cells, expressing GP2 and CUZD1 separately, are applied as substrates and were co-incubated with pre-diluted sera (exactly 1:10, 1:100 and 1:1000) for 30 min. at room temperature. Unbound sample was then washed away and fluorescein-labeled goat anti-human IgA or IgG antibodies were used to visualize bound antibodies of the patients' sera. Evaluation was performed under using a Eurostar Plus microscope with Bluelight LED (Euroimmun Medizinische Labordiagnostika AG). **Supporting Figure 1** shows immunofluorescence staining patterns on HEK293 transfected with either CUZD1 or GP2. According to findings of *Komorowski et al.*⁹ reaction with transfected HEK293 cells expressing CUZD1 entirely corresponds to reticulogranular PAb pattern (type 1)

on frozen sections of the human pancreas, whereas GP2-HEK reaction does the droplet pattern (type 2).

Antibody Assays for anti-*Saccharomyces cerevisiae* antibodies (ASCA)

ASCA antibody evaluation in CD patients was performed by ELISA (QUANTA Lite™, Inova Diagnostics, San Diego, CA) according to the manufacturers' instructions. The results are presented as arbitrary units, and values above the cut-off of 25 units were considered as positive. The results were documented in absolute values and in frequency of positivity.

Detection of NOD2/CARD15 SNP8, 12, 13 mutations

NOD2/CARD15 SNP8, SNP12, and SNP13 genotypes were performed previously³¹ in CD patients (n=235), but not in UC patients. NOD2/CARD15 variants were detected 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). All investigated polymorphisms were in Hardy–Weinberg equilibrium (data not shown).

Ethical considerations

The regional and national committee [DEOEC RKEB/IKEB 3515-2011, 3880/2012/EKU (59/PI/2012)] for research ethics approved the study protocol. Each patient was informed of the nature of the study and signed an informed consent form.

Statistical analysis

Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as means [standard deviation (SD)] or as medians [interquartile range (IQR)] according to their homogeneity. To evaluate differences between IBD and healthy control group, as well as within subgroups of patients with IBD, the following statistical methods were used. Categorical variables were compared with the Fisher's exact test or χ^2 -test with Yates correction, linear-by-linear association, as appropriate. Continuous variables were compared with Student's t test, one-way analysis of variance (ANOVA), or Mann-Whitney's U test or Kruskal-Wallis H test with Post-hoc analysis (Dunn's multiple comparison test). Kaplan-Meier survival curves were plotted for analyzing the association between categorical clinical variables or serological antibodies and complicated disease outcomes during follow-up with LogRank test or Cox-regression analysis in the time-dependent models. Associations are given as odds ratio (OR) and hazard ratio (HR) with a 95% confidence intervals (CI). A 2-sided probability value <0.05 was considered to be statistically significant. For statistical analysis, GraphPadPrism 6 (San Diego, CA) and SPSS 22.0 (SPSS Inc, Chicago, IL) programs were used.

RESULTS

Frequency of anti-pancreatic antibodies in IBD

The frequency of the different antibodies in IBD and controls is summarized in **Table 2**. Significantly more, 10.2%, 12.2%, 10.2% and 20.8% of the CD patients were positive for anti-GP2 IgA/IgG, anti-MZGP2 IgA/IgG, anti-rPAg2 IgA/IgG and also for anti-CUZD1 (\approx anti-rPAg1) IgA/IgG antibodies compared to both UC and healthy controls ($p < 0.01$ for all), respectively. In addition, significant differences were found for both IgA and IgG subtypes. In contrast, the positivity rate was not different in UC and controls.

Anti-GP2 and anti-MZGP2 antibody titers were higher in CD compared to UC. The difference of the median titers for all antibodies, except anti-MZGP2 IgA, between CD and UC was statistically significant (Mann-Whitney, anti-GP2 IgA $p < 0.001$, anti-GP2 IgG $p < 0.001$, anti-MZGP2 IgA $p = 0.12$, anti-MZGP2 IgG $p < 0.001$).

Correlation of anti-pancreatic antibodies formation and overall disease duration in Crohn's disease

Stability of anti-pancreatic antibodies in Crohn's disease

No association was detected between antibody status and clinical or endoscopic disease activity (actual HBI or SES-CD) at the time of sample procurement (data not shown). In addition, to evaluate the stability of PAb status (positive or negative for a respective antibody), we analyzed samples from same patients over various arbitrary time-points during the disease course. At least two serum samples were taken from

the majority of CD patients (n=192) and re-tested for all the different PAb. Median time between sample procurements was 30.3 months [IQR, 15.3-48.7]. Interestingly, the status of different PAb was very stable over time regarding both IgA and IgG subtypes with only $\leq 5\%$ of cases changing their PAb status over time. Stability data of PAb are summarized in **Supporting Information Table 2**. Development of disease complications or surgical procedure did also not affect the antibody status of any of the PAb (data not shown).

Predictive potential of anti-pancreatic antibodies for disease outcomes in Crohn's disease

We analyzed the association of the different PAb markers with poor disease outcomes (development of internal penetrating and/or stricturing disease, perianal perforating disease and need for first and subsequent surgical resection) in time-dependent univariate models.

None of the PAb markers were able to predict the development of internal penetrating and/or stricturing complications in patients with inflammatory disease behavior (B1) (n=211).

Among patients without previous perianal complication (any behavior) (n=216), those who were positive for anti-CUZD1 IgA/IgG more likely progressed to develop perianal complications (pLogRank=0.008). The association was stronger for the IgA antibody subtype (pLogRank<0.001) and a quantitative association was also found with IgA antibody titers (pLogRank<0.001) (**Figure 3**). The sensitivity analysis performed in patients with B1-only behaviour (n=169) after excluding patients with an initial B2/3 phenotype yielded the same results regarding the development of

perianal complications (pLogRank<0.001 for the anti-CUZD1 IgA).

In the surgery-naïve patient group (n=234), progression to first resective surgery was faster in patients positive for anti-GP2 IgA antibody (pLogRank=0.002) (**Figure 2**). At the same time, in postoperative setting, the presence of anti-GP2 IgA antibody was not able to predict a subsequent resective surgery in patients with prior surgery (n=109).

To further evaluate the predictive potential of the PAb markers, we used a Cox-proportional Hazard regression model adjusted to gender and clinical variables with a p value of < 0.1 in univariate time dependent models (Kaplan-Meier and Log-rank analysis). Anti-CUZD1 IgA and anti-GP2 IgA were analyzed separately. Anti-CUZD1 IgA was identified as an independent predictor for the development of perianal disease both in the primary model(**Table 4**)and also in the model in the same sensitivity analysis (data not shown).In contrast, the association between anti-GP2 and need for resective surgery was lost in the multivariate model (**Table 4**).

Association between anti-pancreatic antibody positivity and clinical, serologic and genetic characteristics of Crohn's disease

Anti-MZGP2 IgA/IgG antibodies were more prevalent in patients with pediatric disease onset (A1: 28.6%, A2:11.3%, A3:5.7%, p=0.012)and anti-rPAg2 IgA/IgG antibodies (L1/3 vs. L2, 13.2% vs. 4.5%, p=0.032) were more frequent in patients with ileal involvement. Penetrating disease behavior at last follow-up was associated with anti-GP2 IgA/IgG (17.9% vs. 7.3%, p=0.026), anti-MZGP2 IgA/IgG (20.2% vs. 8.9%, p=0.040) and anti-rPAg2 IgA antibodies (13.1% vs. 4.8%, p=0.040).

In addition, anti-GP2 IgA/IgG (14.5% vs. 6.6%, $p=0.036$) was associated with need for resective surgery at maximum follow-up. Of the extraintestinal manifestations, anti-rPAg2 IgA/IgG positivity was associated withPSC (37.5% vs. 9.4%, $p=0.038$). In contrast, PAb directed against CUZD1 (\approx anti-rPAg1) was associated withcolonic involvement (L2/L3 vs. L1, 23.7% vs. 10.5%, $p=0.041$ for IgA/IgG subtype), perianal diseaseat maximum follow-up (P1 vs. P0, 32.6% vs. 15.0%, $p=0.001$ for IgA/IgG subtype) and cutaneous manifestations(23.5% vs. 10.4%, $p=0.044$ for IgA subtype). **Table 3** presents the associations between antibody reactivities to different PABs and diseases characteristics in patients with CD at diagnosis and maximum follow-up according to Ig subtypes. There was an association between the presence of IgA type PABs and ASCA antibodies. The different PAB IgA antibodies were significantly more frequent in ASCA IgA positives compared to negatives (anti-GP2 IgA: 6.7% vs. 0.0%, anti-MZGP2 IgA: 14.6% vs. 1.7%, anti-rPAg1: 17.5% vs. 4.9% and anti-rPAg2: 11.7% vs. 2.4%, $p < 0.01$ for all). However, PAB IgG positivity did not differ significantly according to presence or absence of ASCA IgG (**Table 3**). The prevalence of different PABs was also not associated with the presence or absence of major NOD2/CARD15 mutations (data not shown).

Interassay study for anti-GP2 antibodies

Altogether, the agreement among the three different assays ranged from 94.0% to 95.6% for anti-GP2 IgG and from 91.6% to 96.4% for anti-GP2 IgA. The κ coefficients suggested good concordance between the different assays. The exact κ values are summarized in **Supporting Information Table 1**.

DISCUSSION

In the present study, we investigated the clinical importance of different target specific PABs in the prediction of complicated diseases behavior and surgery in adult CD patients. To our knowledge, this is the first prospective study on the new PABs. In addition, we proved the long-term stability of PAB status over time and confirmed good agreement among newly available diagnostic tools for evaluation of anti-GP2 antibodies.

Significantly more CD patients were positive for different PABs compared to both UC patients and healthy controls. The formation of autoantibodies against GP2 and CUZD1 glycoproteins may reflect an immune response against an overwhelmed microbial challenge to the intestinal barrier. Anti-GP2 antibody titers were higher in CD compared to UC as well. The magnitude of antibody responses (titers) may correlate with the extent of bacterial translocation (BT), which is more pronounced in CD compared to UC. One possible explanation is that in UC the inflammation is confined to the mucosa whereas in CD it is transmural. Another explanation might be that the disturbance of mucosal immunity is different between the two diseases. It is further supported by the findings that the presence of GP2 was confirmed at the intestinal site of inflammation in patients with CD³².

In previous clinical studies, presence of anti-GP2 antibodies was associated with distinct clinical phenotype of CD such as early disease onset, ileal involvement and stricturing disease behavior but results differed among studies. However, in the single study identifying anti-CUZD1 antibody as a distinct PAB type the association with clinical phenotype or disease course has not been assessed in CD⁹. Interestingly, clinical associations were different for anti-GP2 and anti-CUZD1 antibodies in the present cohort, which may be at least partly the consequence of differences in the

target proteins of these antibodies belonging to distinct innate immunity protein family. Prevalence of anti-GP2 antibodies was higher in our patients with pediatric onset in concordance with findings of some previous studies^{14, 15}. Regarding disease location, we were also able to confirm that presence of anti-GP2 antibodies was associated with extensive disease with ileal involvement¹⁴⁻¹⁶. Of note, some reports failed to identify an association between anti-GP2 antibodies and disease location³³ or this was limited to only upper gastrointestinal tract involvement¹⁸. Anti-CUZD1 antibodies were more frequent in patients with colonic involvement. Penetrating disease was associated with anti-GP2 positivity in the present CD cohort. In the study of *Bogdanos et al.*¹⁵ anti-GP2 IgG were significantly more prevalent in patients with stricturing behavior and perianal disease, but less prevalent in those with penetrating behavior, while other studies did not find significant association between anti-GP2 antibodies and complicated disease behavior^{14, 18}. Thus far, no studies have evaluated associations between target specific PAbs and extraintestinal manifestations. We found that PSC was associated with the presence of anti-GP2 while ocular manifestations were associated with anti-CUZD1 antibodies. Of note, despite these differences between the clinical phenotypes the concordance among the three assays was good. Each test recognized also an additional patient population that was missed by the other(s), suggesting partly non-overlapping epitopes.

Our findings support that PAbs may contribute to better stratification of CD patients. Prevalence is relatively low and so the clinical utility of PAbs in the diagnosis and prediction disease course in CD may be more modest compared to ASCA which remains the most accurate single marker in CD so far². However, anti-GP2 antibody has been shown to be more specific for CD than ASCA recently and the

specificity of double positive patients with CD is 100%¹⁴. Of note, the prevalence rate of anti-GP2 IgA/IgG in our CD patients was even lower than those ones reported previously (10.2-12.4% vs. 21.0-45.0%)³⁴. Variation in the prevalence of serologic markers among studies and in different ethnic populations is however well documented². Moreover methodological differences can also contribute to these differences. It is interesting to note that different isoforms of GP2 are synthesized in the pancreas³⁵. A larger and a shorter isoform of GP2 were identified and termed them alpha (GP2a) and beta (GP2b), respectively³⁶. Previous prevalence data of anti-GP2 IgA and IgG, except one¹⁴, were obtained mainly by assays manufactured by GA Generic Assays employing the larger isoform for GP2-specific autoantibody detection initially¹³. In contrast, both ELISAs used in our study employed recombinant human GP2 isoform 4 as solid-phase antigen corresponding to the shorter isoform (GP2b) due to better discrimination of patients with CD from those with UC³⁵.

For the long-term predictive potential of serologic markers it is necessary to assess the stability of antibody status over time. In terms of anti-GP2 antibody stability, only limited and conflicting data are available¹⁷. In the present study long-term stability of different PABs was assessed extensively. PAB status was not associated with actual disease activity and positivity rates were stable over time in IBD patients. This is in contrast to anti-GP2 antibody levels detected in patients with celiac disease where they were significantly reduced under a gluten-free diet³.

Until now, only cross-sectional associative analyses are available in CD patients. In addition, the present study provides longitudinal prospective results on the predictive potential of PABs for identifying disease specific complications and surgery requirements. PAB positivity was able to predict faster progression to complicated disease in our patient cohort. Anti-GP2 positivity was associated with the need for

surgical interventions.while anti-CUZD1 predicted development of perianal complications.Regarding anti-GP2, previously only two cross-sectional studies evaluated surgical outcome with conflicting results^{16, 18}Of note however, treatment strategy may vary among countries, IBD centres and even doctors which is a clear confounder of all studies. Thereforeassociation between serology and surgical findings in the present study should be interpreted with caution.. However, one of the main strengths of our study is the analysis of outcomes during a prospective follow-up period. The frequency of regular follow-up was 3-6 months in the majority of the patients. Silverberg et al.²⁰ suggested that long-term complications and outcomes should be studied during an approximate 5-year follow-up. Of note, 79.2% of the CD patients in the present cohort had at least a 5-year follow-up. In addition, the collection of extensive disease phenotypes enabled adjusting for possible confounders. In the final Cox-regression multivariate model, anti-CUZD1 antibody positivity was a strong independent predictor for the development of perianal complicationsincluding age at diagnosis, sex, disease location and behavior and relapse frequencyas potential confounders.Our results await replication in independent patient cohorts both from referral centers and population-based cohorts.

Interestingly, in our study IgA,but not IgG PABs typeswere associated with complicated diseases course in CD.The gut mucosal immune system plays a central role in the IgA antibody formation and this may at least partly reflect an immune response against an overwhelmed microbial challenge.In addition, IgA type autoantibodies are considered a sign of immunological response to enteric antigens in other diseases associated with enhanced bacterial translocation.Moreover IgA type antibodies were reported to have a pivotal role in the development of disease-specific complicationscompared to the IgG antibody subtype³⁷.Our clinical findings are

concordance with a previous hypothesis from Roggenbuck *et al.*^{12, 17}. According to this hypothesis, plasma cells can synthesize anti-GP2 IgA after the loss of tolerance towards GP2, which will be actively transported by the epithelium into the intestinal lumen. Indeed, anti-GP2 IgA has been found in faeces of patients with pouchitis exhibiting CD-like complications³⁸. The secreted anti-GP2 IgA can bridge FimH positive bacteria opsonised by pancreatic GP2 with the membrane-bound GP2 on the M cells. Ultimately, this may lead to a mucosal overload of microbes in CD due to elevated transcytosis accelerating intestinal inflammation.

In conclusions, the findings of our prospective referral cohort study indicate that target-specific PAbs may be useful markers in the stratification of CD patients and are associated with complicated disease phenotype and risk of developing perianal complications. In addition, they may be valuable additional tools as a member of serology panels for the prediction of disease course.

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FIGURE LEGENDS

Figure 1 Flow chart of the patients with Crohn's disease (CD) in the cohort study. Event: complication and/or surgery; Complication: stricture development and/or internal penetration and/or perianal penetration; Surgery: CD-related surgery (resection only). Pts: patients

Figure 2 Kaplan-Meier survival analysis for the probability of the perianal disease development

Figure 3 Kaplan-Meier survival analysis for the probability of the resective surgery

TABLES

Table 1 Clinical characteristics of IBD patients

Table 2 Serologic markers in patients with Crohn's disease (CD), ulcerative colitis (UC), and healthy controls (HCONT).

Table 3 Associations between antibody reactivities to different PABs and diseases characteristics in patients with Crohn's disease at diagnosis (A) and last follow up (B). Positive associations are indicated in bold and negative associations in italic (p values, odds ratio and 95%CI).

Table 4 Summary of Cox Proportional Hazard Regression Model: factors affecting time to development of perianal disease (A) and need for resective surgery (B) in patients with Crohn's disease.

Table 1 Clinical characteristics of IBD patients

	CD (n= 271)	UC (n= 187)
Male/female (n)	115/156	86/101
Age at presentation (years)*	25 (19-33)	33 (23-43)
Follow-up (months)*[□]	108 (65-178)	104 (62-182)
Familial IBD¹	12 (4.4%)	6 (3.2%)
Location/extent at diagnosis¹		
L1	60 (22.1%)	proctitis 30 (16.0%)
L2	88 (32.5%)	left-sided 104 (55.6%)
L3	122 (45.0%)	extensive 53 (28.3%)
L4 only	1 (0.4%)	
All L4	16 (5.9%)	
Behavior at diagnosis¹		
B1	216 (79.7%)	
B2	33 (12.2%)	
B3	22 (8.1%)	
Perianal disease¹		
at diagnosis	49 (18.1)	
last follow-up[□]	93 (35.1)	
Cumulative exposure of medication and surgeries during follow-up		
Steroid use/refractory¹	239 (88.2%) / 31 (13.0%)	144 (77.0%) / 11 (7.6%)
Azathioprine use¹	200 (73.8%)	66 (35.3%)
Surgery/multiple in CD colectomy in UC¹	109 (41.1%) / 31 (11.7%)	7 (3.7%)
Biological use¹	113 (41.7%)	25 (13.4%)
Smoking habits¹		
never	219 (80.8%)	167 (89.3%)
yes	47 (17.3%)	18 (9.6%)
previous	5 (1.8%)	2 (1.1%)

[□]: 265 CD patients had follow-up

¹n (%), *: median (IQR)

Location: L1: ileal, L2: colonic, L3: ileocolonic, L4: upper gastrointestinal disease

Behavior: B1: non-stricturing/ non-penetrating, B2: stenosing, B3: penetrating

Surgery: CD-related abdominal surgery and colectomy in UC

Table 2. Serologic markers in patients with Crohn's disease (CD), ulcerative colitis (UC), and healthy controls (HCONT)

	CD Anti-GP2 n=266 Anti-MZGP2 n=266 Anti-rPAG n=264	UC Anti-GP2 n=183 Anti-MZGP2 n=185 Anti-rPAG n=178	HCONT Anti-GP2 n=100 Anti-MZGP2 n=100 Anti-rPAG n=100
Anti-GP2 IgG	27 (10.2%) ^{□***}	3 (1.6%) [□]	1 (1.0%) ^{**}
Anti-GP2 IgA	9 (3.4%) [†]	0 (0.0%) [†]	1 (1.0%)
Anti-GP2 Either	27 (10.2%)^{□*}	3 (1.6%)[□]	2 (2.0%)[*]
Anti-MZGP2 IgG	26 (9.8%) ^{□§}	2 (1.1%) [□]	2 (2.0%) [§]
Anti-MZGP2 IgA	22 (8.3%) [□]	0 (0.0%) [□]	4 (4.0%)
Anti-MZGP2 Either	33 (12.4%)[□]	2 (1.1%)[□]	6 (6.0%)
Anti-rPAG1 IgG	43 (16.3%) [⌘]	12 (6.7%)	0 (0.0%) [⌘]
Anti-rPAG1 IgA	32 (12.1%) [⌘]	11 (6.2%)	0 (0.0%) [⌘]
Anti-rPAG1 Either	55 (20.8%)^{‡,⌘}	16 (9.0%)[‡]	0 (0.0%)[⌘]
Anti-rPAG2 IgG	18 (6.8%) ^{‡,**}	0 (0.0%) [‡]	0 (0.0%) ^{**}
Anti-rPAG2 IgA	19 (6.9%) ^{‡,**}	1 (0.6%) [‡]	0 (0.0%) ^{**}
Anti-rPAG2 Either	27 (10.2%)^{□□}	1 (0.6%)[□]	0 (0.0%)[□]
Anti-rPAG IgG	55 (20.8%) ^{□⌘}	12 (6.7%) [□]	0 (0.0%) [⌘]
Anti-rPAG IgA	46 (17.4%) ^{⌘,⌘}	12 (6.7%) [⌘]	0 (0.0%) [⌘]
Anti-rPAG Either	73 (27.7%)^{□⌘}	17 (9.6%)[□]	0 (0.0%)[⌘]

Cut-off levels were 25 U/ml for anti-GP2, 20 U/ml for anti-MZGP2 by enzyme-linked immunosorbent assay and 1:10 titer for anti-rPAG antibodies by indirect immunofluorescence assay.

Anti-rPAG1 and rPAG2 corresponds to anti-CUZD1 and anti-GP2, respectively.

CD vs. controls: § $p=0.015$, * $p=0.01$, ** $p<0.01$, □ $p=0.001$, ⌘ $p<0.001$,

CD vs. UC: † $p=0.01$, # $p<0.01$, ‡ $p=0.001$, □ $p<0.001$

Using χ^2 -test with Yates correction.

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(B)

	Anti-GP2			Anti-MZGP2			Anti-rPAG2 (\approx anti-GP2)			Anti-rPAG1 (\approx anti-CUZD1)		
	IgA	IgG	IgA and/ or IgG	IgA	IgG	IgA and/ or IgG	IgA	IgG	IgA and/ or IgG	IgA	IgG	IgA and/ or IgG
Perianal disease										0.001 3.59 (1.66–7.74)	0.009 2.45 (1.26–4.76)	0.001 2.75 (1.50–5.05)
Stricturing												
Penetrating	0.032 5.55 (1.12–27.40)	0.026 2.78 (1.15–6.69)	0.026 2.78 (1.15–6.69)	0.013 3.60 (1.31–9.90)		0.023 2.61 (1.15–5.90)	0.040 2.96 (1.05–8.36)					
Need for resective surgery	0.036 5.20 (1.06–25.5)	0.036 2.47 (1.07–5.68)	0.036 2.47 (1.07–5.68)									
Cutaneous Manifestation										0.044 2.64 (1.08–6.48)		
PSC							0.014 9.00 (1.97–41.08)		0.038 5.80 (1.31–25.78)			

Table 4 Summary of Cox Proportional Hazard Regression Model: factors affecting time to development of perianal disease (A) and need for resective surgery (B) in patients with Crohn's disease. Positive associations are indicated in bold

p value: level of significance; 95% CI: 95% confidence interval, NSNP: non-stricturing non-penetrating

A

	<i>p</i> value	Hazard Ratio	95% CI
Female gender	0.275	0.70	0.36-1.33
Age at onset		0.13	
> 40 yrs		Reference	
17-40 yrs	0.088	5.84	0.77-44.36
≤ 16 yrs	0.049	8.42	1.01-70.39
Frequent relapse	< 0.001	3.50	1.78-6.89
Colon involvement	0.044	4.41	1.04-18.73
Behaviour		0.267	
NSNP		Reference	
Stricturing	0.368	1.58	0.58-4.30
Penetrating	0.137	2.65	0.73-9.59
Anti-CUZD1 IgA positivity	< 0.001	3.67	1.77-7.62

B

	<i>p</i> value	Hazard Ratio	95% CI
Female gender	0.970	0.99	0.66-1.49
Age at onset		0.813	
> 40 yrs		Reference	
17-40 yrs	0.540	0.84	0.47-1.48
≤ 16 yrs	0.821	0.91	0.40-2.05
Ileal involvement	0.129	1.44	0.90-2.32
Behaviour		< 0.001	
NSNP		Reference	
Stricturing	< 0.001	5.45	3.27-9.10
Penetrating	< 0.001	12.48	6.69-23.28
Anti-GP2 IgA positivity	0.225	1.65	0.73-3.73

Figure 1 Flow chart of the patients with Crohn's disease (CD) in the cohort study.

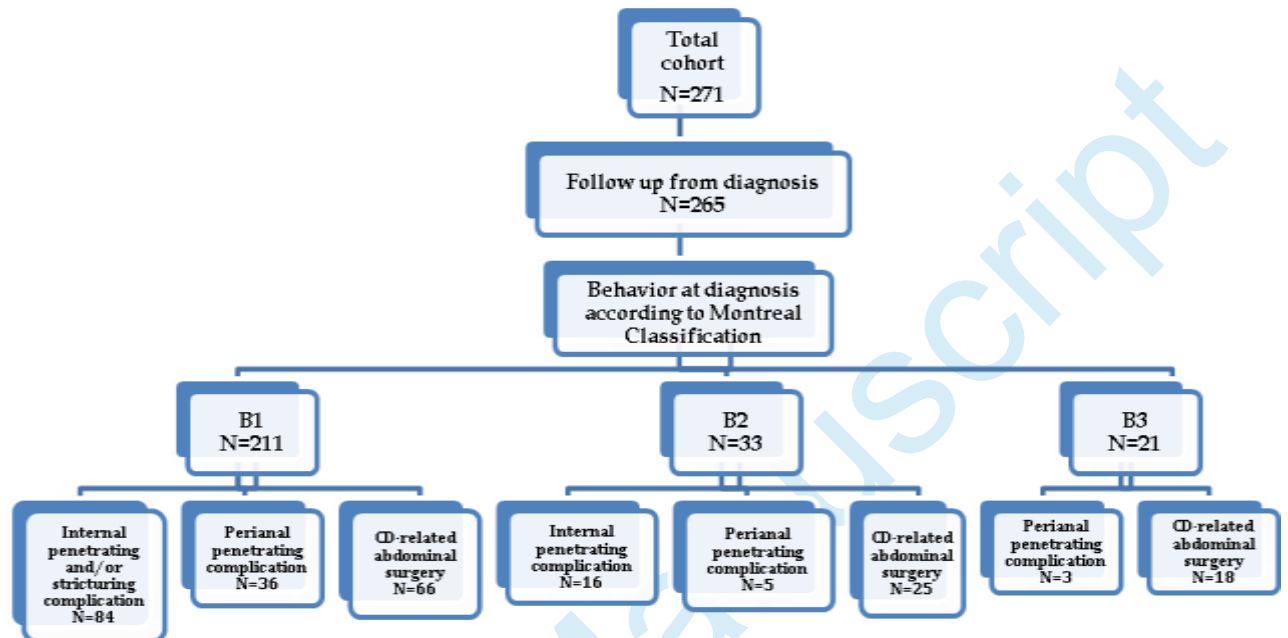


Figure 2 Kaplan-Meier survival analysis for the probability of the resective surgery

NSNP: non-stricturing non-penetrating

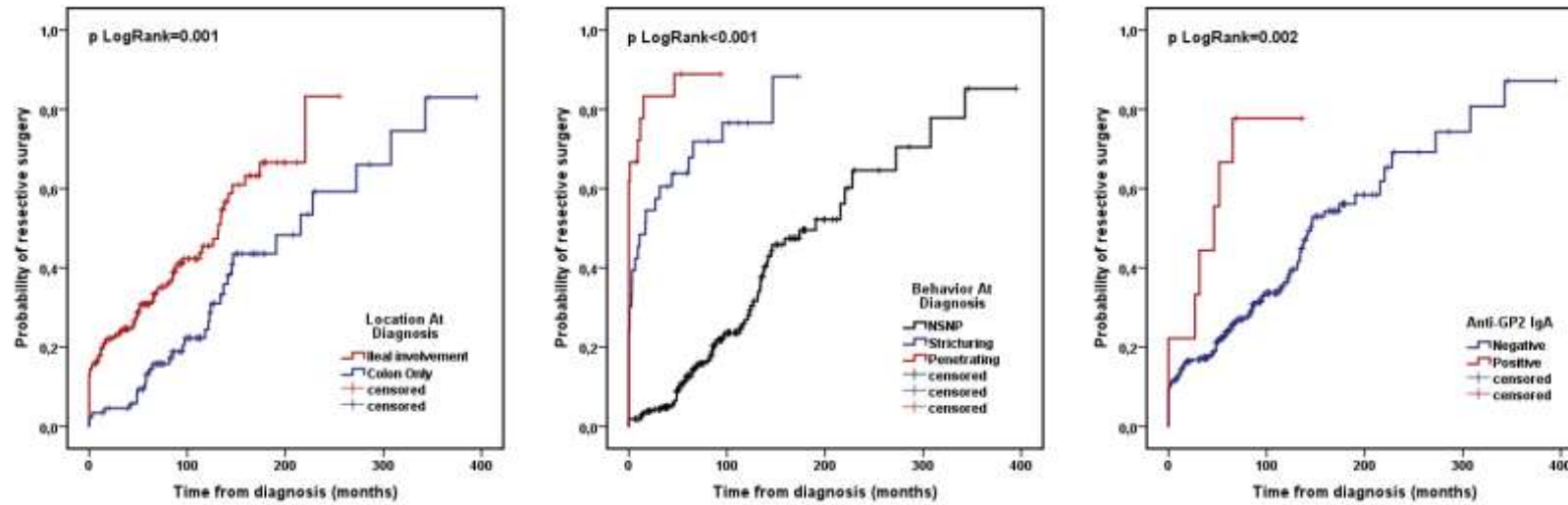


Figure 3 Kaplan-Meier survival analysis for the probability of the perianal disease development

