Antibodies against glycoprotein 2 display diagnostic advantages over ASCA in distinguishing CD from intestinal tuberculosis and intestinal Behçet's disease

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OBJECTIVES: There is an increasing need to identify reliable biomarkers for distinguishing Crohn’s disease (CD) from other gastrointestinal disorders sharing similar clinical and pathological features. This study aimed at evaluating the diagnostic potential of antibodies to zymogen granule glycoprotein GP2 (aGP2) in a large, well-defined Chinese cohort with a special focus on their role in discriminating CD from intestinal Behçet’s disease (BD) and intestinal tuberculosis (ITB).

METHODS: A total of 577 subjects were prospectively enrolled, including 171 patients with CD, 208 patients with ulcerative colitis (UC), 71 with BD, 57 with ITB and 70 healthy controls (HC). aGP2 and anti-Saccharomyces cerevisiae antibodies (ASCA) were determined by ELISA. Perinuclear antineutrophil cytoplasmic antibodies were tested by indirect immunofluorescent assay.

RESULTS: aGP2 IgG and IgA levels were significantly elevated in patients with CD compared with those in patients with UC, intestinal BD, and ITB and HC. Conversely, ASCA IgG levels were not different between CD and intestinal BD patients, whereas ASCA IgA levels did not discriminate CD from intestinal BD and ITB patients. aGP2 IgA and IgG displayed a better assay performance (larger areas under the curve) over ASCA IgA and IgG in differentiating CD from disease controls (P < 0.05). ASCA IgA did not discriminate CD from disease controls. aGP2 IgA and/or IgG was significantly associated with penetrating disease (B3) and ileal CD (L1) (P < 0.05), whereas ASCA IgA and/or IgG was not.

CONCLUSIONS: In comparison with ASCA, aGP2 distinguishes CD from intestinal BD or ITB as disease controls more efficiently, aiding in the differential diagnosis of IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn’s disease (CD) and ulcerative colitis (UC), represents a group of chronic inflammatory disorders in the gastrointestinal (GI) tract.1,2 IBD have multifactorial etiology, which includes dysregulation of the immune response to the gut microbiota, genetic susceptibile polymorphism and environmental risk factors.3

Currently, the diagnosis of CD remains a tremendous challenge to physicians, and most patients with CD suffer from a diagnostic delay (a period from appearance of first symptoms to diagnosis), which may lead to a complicated disease course and increased operation rate.4 As CD covers a series of rather non-specific clinical symptoms including intestinal and extra-intestinal involvements, other disorders affecting the GI with similar clinical manifestations create a diagnostic dilemma. Particularly, intestinal tuberculosis (ITB) and intestinal Behçet’s disease (BD) often present similar clinical symptoms and pathology to those seen in CD patients.5,6 In addition, subclassification of IBD into CD and UC poses another diagnostic dilemma. CD and UC display considerable differences in terms of lesion localization in the GI and histopathologic presentations,1,2 resulting in significant differences in clinical management and therapy options. Furthermore, stratification of 10–15% of all IBD patients is also challenging in the case of indeterminate colitis due to undiscriminating biopsy data and a certain overlap of colonic inflammation symptoms in IBD patients.7,8

Serological biomarkers have gained extensive attention over the past decade due to their ready availability and
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Non-invasiveness, and, hence, have been used complementary to endoscopic and histological tests. Anti-saccharomyces cerevisiae antibodies (ASCA) and perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) are routinely utilized for screening patients with clinical suspicion of IBD. However, it has been suggested that the discriminatory capability of ASCA in CD is far from satisfactory due to its unsatisfactory sensitivity and specificity, especially in Asia. For example, studies from our group and others have found that ASCA displayed a poor clinical performance in differentiating CD from ITB, highlighting a critical need for developing new biomarkers.

In light of recent research on the antigenic targets of pancreatic autoantibodies (PAB), zymogen granule glycoprotein 2 (GP2) stood out as a major autoantigen of PAB. GP2, which is a highly glycosylated protein with two major sites of synthesis (pancreatic, intestinal), is overexpressed at the site of intestinal inflammation in patients with CD, indicating a direct involvement of GP2 in the inflammatory process. In fact, anti-GP2 antibodies (aGP2) were present in 21–45% patients with CD and significantly less prevalent in patients with UC. Of interest, ASCA-negative CD patients were tested positive for aGP2 IgA and/or IgG, highlighting the potential of aGP2 in the serological diagnosis of CD. We have previously demonstrated that IgA/IgG aGP2 were present in 54.3% of Chinese patients with CD and just 14.3% with UC in small cohorts of patients with IBD (35 CD and UC patients, each), supporting the aforementioned diagnostic potential of aGP2. However, the small sample size may have introduced analytic bias and, thus, a larger cohort study is badly needed. Furthermore, to our knowledge, no study has assessed the clinical relevance of aGP2 in distinguishing CD from ITB and intestinal BD. Given the high prevalence of intestinal BD or ITB in China and other Asian countries as well as the diagnostic challenge of differentiating those two diseases from CD, it is of paramount importance to assess the clinical significance of aGP2 accordingly. Thus, in the present study, we included a large cohort of IBD patients, including 171 patients with CD and 208 with UC as well as 128 disease controls (71 patients with intestinal BD and 57 patients with ITB) for the evaluation of the diagnostic performance of aGP2.

Materials and Methods

Subjects and disease stratification. A total of 577 subjects were prospectively enrolled in this study, including 171 patients with CD, 208 patients with UC, 71 patients with intestinal BD, and 57 patients with ITB and 70 healthy controls (HC). HC included subjects without any signs of infection or inflammation or other apparent illnesses. All patients were diagnosed and managed at the Department of Gastroenterology, Peking Union Medical College Hospital (PUMCH). The diagnosis of IBD was determined based on the Lennard-Jones criteria. Accordingly, subjects were diagnosed with CD or UC based on a combination of standard criteria that included clinical symptoms, physical examination, colonoscopy, imaging (bariums studies and CT enterography), and histopathology. Patients with enteric infections, ischemia, non-steroidal anti-inflammatory drug induced ulceration, and radiation colitis were excluded. Clinical phenotypes of IBD patients were determined based on the Montreal Classification (age at diagnosis: A1, below 17 years; A2, between 17 and 40 years; A3, above 40 years, location of disease: L1, ileal; L2, colonic; L3, ileocolonic; L4, upper disease, disease behavior: B1, non-strictureing, non-penetrating; B2, strictureing; B3, penetrating; P, perianal disease modifier). Patients with UC were classified by classification (E1, proctitis, lesions limited to the rectum; E2, left-sided colitis, lesions below the splenic flexure; E3, pancolitis, lesions exceeded the splenic flexure). The activity of UC was defined by the Simple Clinical Colitis Activity Index (SCCAI) as mild (3–5 scores), moderate (6–11 scores) and severe (above 12 scores). The activity of CD was defined by Crohn's Disease Activity Index (CDAI), as previously described with CDAI scores < 150 as symptomatic remission and CDAI scores ≥ 150 as active disease. Specifically, the activity of CD was defined as mild (CDAI scores of 150–220), moderate (CDAI scores of 221–450) and severe (CDAI scores of > 450). The demographics and clinical characteristics of the CD and UC patients are shown in Table 1. Study protocols were reviewed and approved by the Ethical Committee of PUMCH and informed consents were obtained from all participants. All sera were stored at −20 °C until analysis.

Serum antibodies determination. Serum aGP2 IgG and IgA were determined by ELISA (Generic Assays, Dahlewitz/Berlin, Germany), according to the manufacturer's instructions. The cutoff value for positivity was set to 15 U/ml for IgG aGP2 and 10 U/ml for IgA aGP2, as recommended by the manufacturer. Serum IgG ASCA and IgA ASCA were determined by ELISA (Inova Diagnostics, San Diego, USA). Values above 25 U/ml were considered as positive according to the manufacturer's instructions. Serum IgG pANCA and IgA pANCA were tested by indirect immunofluorescent assay (IFA) (Euroimmune, Luebeck, Germany), in accordance with the manufacturer's instructions. IFA testings were performed starting with an initial dilution of 1/10. Serial dilutions of 1/20, 1/40, 1/80, and 1/160 were further performed for all positive samples. Two experienced technologists interpreted the results.

Discriminatory capability of aGP2 and ASCA in differentiating CD vs. UC and CD vs. disease controls. Receiver operating characteristics (ROC) analysis was utilized to evaluate the discriminatory capability of aGP2 and ASCA in differentiating CD vs. UC and CD vs. disease controls. ROC curves were generated by plotting sensitivity vs. (1-specificity) for IgG aGP2, IgA aGP2, IgG ASCA and IgA ASCA. Areas under the curves (AUCs) with their corresponding 95% confidence intervals (CIs) were determined.

Statistical analysis. All statistical tests were performed by SPSS 20.0 statistical software package (SPSS Inc., Chicago, Illinois, USA), Prism 5.02 (GraphPad Software, San Diego, California, USA) and MedCalc (MedCalc Software, Ostend, Belgium). Quantitative variables were compared with a Kruskal-Wallis test followed by
post-hoc analysis by Conover. Categorical variables were compared with a χ² test or Fisher’s exact testing for 4 × 4 contingency tables. The clinical relevance of multiple antibodies was assessed with logistic regression models for each clinical variable, and the results are presented as odds ratio with 95% CI. Receiver operator curves (ROCs), which were constructed by logistic regression models, as previously described, were used to determine the discrimination power of aGP2 and ASCA. Spearman’s rank correlation coefficient was used to assess the

Table 1: Demographics of patients with inflammatory bowel disease and controls

|               | CD (n = 171) | UC (n = 208) | Intestinal BD (n = 71) | ITB (n = 57) | HC (n = 70) |
|---------------|--------------|--------------|------------------------|--------------|-------------|
| Female, n (%) | 45 (26.3)    | 95 (45.7)    | 32 (45.1)              | 34 (59.6)    | 38 (54.3)   |
| Median age at study (years, max, min) | 33 (65, 10) | 43 (77, 12) | 38 (73, 10) | 43 (76, 14) | 45.5 (70, 19) |
| Median duration (years, max, min) | 5 (39, 0.1) | 4 (40, 0.1) | 3.5 (24, 0.1) | 1 (20, 0.1) | N.A.        |
| Median age at diagnosis (years, max, min) | 27 (69, 7) | 36 (70, 12) | 31 (70, 9) | 34.5 (67, 18) | N.A.       |

Age at diagnosis, n (%)

Below 17 years (A1) | 29 (17.0) | 9 (4.3) | 9 (12.7) | 0 (0) | N.A.        |
Between 17 and 40 years (A2) | 105 (61.4) | 117 (56.3) | 31 (43.7) | 34 (59.6) | N.A.        |
Above 40 years (A3) | 37 (21.6) | 82 (39.4) | 31 (43.7) | 23 (40.4) | N.A.        |

Disease location, n (%)

Proctitis (E1) | N.A. | 8 (3.8) | N.A. | N.A. | N.A.        |
Left-sided colitis (E2) | N.A. | 49 (23.6) | N.A. | N.A. | N.A.        |
Pancolitis (E3) | N.A. | 151 (72.6) | N.A. | N.A. | N.A.        |
Ileal (L1) | 36 (21.1) | N.A. | 6 (8.5) | 11 (19.3) | N.A.       |
Colonic (L2) | 39 (22.8) | N.A. | 20 (28.2) | 14 (24.6) | N.A.       |
Ileocolonic (L3) | 96 (56.1) | N.A. | 45 (63.4) | 32 (56.1) | N.A.       |
Upper disease, modifier (L4) | 11 (6.4) | N.A. | 9 (12.7) | 0 (0) | N.A.        |

Disease behavior, n (%)

Non-stricturing, non-penetrating (B1) | 54 (31.6) | N.A. | N.A. | N.A. | N.A.        |
Stricturing (B2) | 58 (33.9) | N.A. | N.A. | N.A. | N.A.        |
Penetrating (B3) | 31 (18.1) | N.A. | N.A. | N.A. | N.A.        |
Stricturing and penetrating (B2+B3) | 28 (16.4) | N.A. | N.A. | N.A. | N.A.        |
Perianal disease (p) | 65 (38.0) | N.A. | N.A. | N.A. | N.A.        |

Disease severity, n (%)

Symptomatic remission | 86 (50.3) | 40 (19.2) | N.A. | N.A. | N.A.        |
Mild | 25 (14.6) | 30 (14.4) | N.A. | N.A. | N.A.        |
Moderate | 44 (25.7) | 65 (31.3) | N.A. | N.A. | N.A.        |
Severe | 16 (9.4) | 73 (35.1) | N.A. | N.A. | N.A.        |

Extraintestinal manifestations

Musculoskeletal | 26 (15.2) | 36 (17.3) | N.A. | N.A. | N.A.        |
Dermatologic | 46 (26.9) | 33 (15.9) | N.A. | N.A. | N.A.        |
Ocular | 4 (2.3) | 4 (1.9) | N.A. | N.A. | N.A.        |
Primary sclerosing cholangitis | 1 (0.5) | 2 (1.0) | N.A. | N.A. | N.A.        |
Thrombosis | 1 (0.5) | 3 (1.4) | N.A. | N.A. | N.A.        |

Treatment, n (%)

5-ASA | 100 (58.5) | 161 (77.4) | N.A. | N.A. | N.A.        |
Immunosuppressive | 78 (45.6) | 35 (16.8) | N.A. | N.A. | N.A.        |
Steroids | 106 (62.0) | 132 (63.5) | N.A. | N.A. | N.A.        |
Response | 68 (44.2) | 80 (38.5) | N.A. | N.A. | N.A.        |
Resistant | 15 (14.2) | 24 (11.5) | N.A. | N.A. | N.A.        |
Dependent | 23 (21.7) | 28 (13.5) | N.A. | N.A. | N.A.        |
Previous surgery | 66 (38.6) | 28 (13.5) | N.A. | N.A. | N.A.        |
GM | 0 (0) | 4 (1.9) | N.A. | N.A. | N.A.        |

Anti-TNF therapy

Response | 43 (25.2) | 8 (3.8) | N.A. | N.A. | N.A.        |
Median duration (years, max, min) | 1 (4.5, 0.1) | 0.3 (2.5, 0.1) | N.A. | N.A. | N.A.        |
Resistance | 2 (4.7) | 2 (1.0) | N.A. | N.A. | N.A.        |
Secondary non-response | 8 (18.6) | 1 (0.5) | N.A. | N.A. | N.A.        |

Abbreviations: BD, Behçet’s disease; CD, Crohn’s disease; GMA, granulocyte and monocyte adsorption apheresis; HC, health controls; ITB, intestinal tuberculosis; TNF, tumor necrosis factor; UC, ulcerative colitis; 5-ASA, 5-aminosalicylic acid; N.A., not applicable. 5-ASA therapy is the first-line treatment for UC and CD. For steroids treatment, response referred to the patients who experienced reduced symptoms and GI inflammation after steroids treatments and the reduced disease was maintained throughout the whole treatment period. Resistance referred to patients who experienced a primary lack of drug efficacy in reducing their symptoms after steroids treatments. Dependence referred to the patients who experienced reduced symptoms and GI inflammation after steroids treatments, but the disease relapsed when the steroids were withdrawn. For anti-TNF therapy, response referred to the patients who experienced reduced symptoms and GI inflammation after anti-TNF therapy and the reduced disease was maintained throughout the whole treatment period. Resistance referred to patients who experienced a primary lack of drug efficacy in reducing their symptoms and GI inflammation after anti-TNF therapy. Secondary non-response referred to patients who failed to maintain an initial response due to acquired drug resistance.
correlations between multiple autoantibodies and age at diagnosis. $P$ values of less than 0.05 were considered significant.

RESULTS

Levels and prevalence of CD-related antibodies in patients with CD, UC, intestinal BD, and ITB as well as HC. All CD-related antibodies demonstrated significantly different values in patients with CD, UC, intestinal BD, ITB, and HC (Kruskal-Wallis test: $P<0.05$, respectively). The levels of IgG and IgA aGP2 were significantly elevated in patients with CD, compared to those patients with UC, intestinal BD, and ITB as well as HC (post-hoc analysis, $P<0.05$) (Figure 1a,b). The prevalences of IgG aGP2, IgA aGP2, IgA or IgG aGP2 (IgA/G aGP2), or IgA and IgG aGP2 (IgA+G aGP2) in patients with CD were 42.7, 33.9, 49.7, and 26.9%, respectively, which were significantly higher than those in the remaining subjects (Table 2). The levels of IgG ASCA were significantly elevated in patients with CD, compared to those in patients with UC, intestinal BD, and ITB ($P<0.05$) (Figure 1c). In contrast, the levels of IgA ASCA in patients with CD were only significantly higher compared to those in patients with UC, but not to those in patients with intestinal BD and ITB as well as HC (Figure 1d).

The prevalences of IgG ASCA, IgA ASCA, IgA or IgG ASCA (IgA/G ASCA), or IgA and IgG ASCA (IgA+G ASCA) in patients with CD were 35.7, 28.7, 45.0, and 19.3%, respectively. The prevalence of IgG ASCA was significantly higher in patients with CD than in other subjects (Table 2). In contrast, no significant differences in the prevalence of IgA ASCA, IgA/G ASCA, or IgA and IgA+G ASCA were observed between patients with CD and patients with intestinal BD. In addition, no significant differences in the prevalence of IgA+G ASCA were identified between patients with CD and patients with ITB (Table 2).

Combination of multiple autoantibodies for distinguishing patients with CD from patients with other disorders. To further assess the role of antibodies in distinguishing patients with CD from patients with other diseases, we calculated specificity and likelihood ratio for each of the antibodies (Table 3). For distinguishing CD from intestinal BD, IgG aGP2, IgA aGP2, IgA/G aGP2, or IgA+G aGP2 displayed a better diagnostic performance than their corresponding ASCA counterparts. Specifically, IgA+G aGP2 exhibited the highest LR+ of 5.83, with a sensitivity of 26.9% and a specificity of 95.4% (Table 3). Similarly, IgG aGP2, IgA aGP2, IgA/G aGP2, or IgA+G aGP2 demonstrated a superior diagnostic performance than their corresponding ASCA counterparts in differentiating CD from ITB. Particularly interesting is the high LR+ value of IgA+G aGP2 (LR+, 10.49) with a sensitivity of 26.9% and a specificity of 97.4% (Table 3).

For the discrimination of CD from UC on a single antibody reactivity basis, IgA+G aGP2 exhibited the highest positive likelihood ratio (LR+) of 5.60, with a sensitivity of 26.9% and...
Prevalence of multiple autoantibodies in patients with CD, UC, intestinal BD, ITB and in HC

| Antibody | CD (n=171) | UC (n=208) | Intestinal BD (n=71) | ITB (n=57) | HC (n=107) |
|----------|------------|------------|----------------------|------------|------------|
| ASCA IgG | 35 (20.5)  | 21 (10.1)  | 8 (11.3)             | 2 (3.5)    | 0 (0)      |
| ASCA IgA | 37 (21.7)  | 26 (12.5)  | 12 (17.1)            | 0 (0)      | 0 (0)      |
| ASCA both | 21 (12.3)  | 14 (6.7)   | 8 (11.3)             | 0 (0)      | 0 (0)      |
| GP2 IgG  | 29 (17.0)  | 12 (5.8)   | 4 (5.6)              | 0 (0)      | 0 (0)      |
| GP2 IgA  | 42 (24.6)  | 19 (9.1)   | 10 (14.1)            | 2 (3.5)    | 0 (0)      |
| GP2 both | 13 (7.6)   | 7 (3.4)    | 4 (5.6)              | 0 (0)      | 0 (0)      |
| pANCA IgG| 22 (12.9)  | 81 (39.2)  | 0 (0)                | 0 (0)      | 0 (0)      |
| pANCA IgA| 25 (14.6)  | 84 (40.5)  | 0 (0)                | 0 (0)      | 0 (0)      |
| pANCA both | 10 (5.9)  | 61 (29.3)  | 0 (0)                | 0 (0)      | 0 (0)      |

| Abbreviations: ASCA, anti-Saccharomyces cerevisiae antibody; BD, Behcet’s disease; CD, Crohn’s disease; GP2, anti-zymogen granule glycoprotein 2 antibodies; HC, health controls; ITB, intestinal tuberculosis; pANCA, anti-neutrophil cytoplasmic antibodies. |

Discriminatory capacities of aGP2 And ASCA in differentiating A subgroup of CD with ileal involvement from ITB or from intestinal BD. The diagnostic potential of aGP2 and ASCA in differentiating ileal CD from ITB or from intestinal BD were also evaluated. Generally, the levels and prevalences of IgG aGP2, IgA aGP2 and IgG ASCA, but not IgA ASCA, were significantly higher in patients with ileal CD than those from ITB or intestinal BD (supplementary Figure 1, and supplementary Table 1). IgG GP2 displayed the highest LR+ of 3.66 in differentiating ileal CD from intestinal BD, followed by IgA GP2 (LR+, 3.33). IgA GP2 exhibited the highest LR+ of 3.67 in differentiating ileal CD from ITB, followed by IgG GP2 (LR+, 2.94) (supplementary Table 2).

Relationship between aGP2 and ASCA in CD. The distributions and relationships between aGP2 and ASCA in patients with CD were illustrated by a Venn diagram. Of note, 35.1% (60/171) patients with CD were negative for both antibodies. The remaining 64.9% (111/171) patients were positive for at least one marker. Interestingly, only 29.8% (51/171) patients were positive for both antibodies. Importantly, 19.9% (34/171) ASCA negative CD patients were positive for aGP2, while 15.2% (26/171) aGP2-negative CD patients were positive for ASCA (Figure 2).

Diagnostic power of aGP2 And ASCA in discriminating CD vs. UC and CD vs. disease controls. ROC analysis was utilized to evaluate the diagnostic power of aGP2 and ASCA in differentiating CD vs. ITB, CD vs. intestinal BD and CD vs. UC. For differentiating CD vs. ITB, IgA aGP2 displayed the highest AUC of 0.716, which was significantly higher than that of IgG ASCA and IgA ASCA (Figure 3 and Table 4). IgG aGP2 displayed a AUC of 0.681, which was significantly higher than that of IgA ASCA and IgA ASCA (Figure 3 and Table 4). IgG aGP2 and IgG GP2 showed similar AUC values of 0.679, respectively, which, were significantly higher than those of IgA ASCA and IgA ASCA (Figure 3 and Table 4). For differentiating CD vs. intestinal BD, IgA aGP2 also demonstrated a better discriminatory performance over IgA ASCA (P=0.0113) and a tendency to a better performance over IgG ASCA (P=0.0691) (Table 4).

Clinical relevance of aGP2 and ASCA with disease characteristics in patients with CD. Patients with CD are heterogeneous in terms of disease presentation, location, behavior, extraintestinal manifestations, and response to treatments. The associations of aGP2 and ASCA with those disease characteristics were evaluated in patients with CD (Table 5). Statistical evaluation by χ² test revealed
a significantly positive correlation of IgG aGP2, IgA aGP2, IgA+G aGP2, IgG ASCA, or IgA+G ASCA with a more complicated penetrating disease (B3) (P < 0.05). Consistently, patients that were negative for both markers were less likely to develop B3 behavior (P = 0.009). In addition, IgA ASCA were positively correlated with stricturing disease (B2) (P = 0.018). IgG aGP2 or IgA+G aGP2 were positively correlated with perianal disease modifier (P < 0.02). Of note, IgA aGP2 and IgA/G aGP2 were positively correlated with ileal location (L1) (P = 0.022), while ASCA-/aGP2- were negatively correlated with L1 (P = 0.003). Further, IgA+G ASCA and ASCA+/aGP2- were positively correlated with ileocolonic location (L3) (P < 0.05), whereas IgA aGP2 were negatively correlated with L3 (P = 0.033). We did not identify any significant associations between the positivity of aGP2 and disease activity in patients with CD (data not shown). Interestingly, IgG aGP2 and IgA ASCA were negatively correlated with age at diagnosis, indicating that patients diagnosed at younger age were more likely to have those

| Table 3 Assay performance parameters of IBD-related antibodies |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CD vs. UC       | Sensitivity (%) | Specificity (%) | PPV (%)         | NPV (%)         | LR+             | LR-             |
| ASCA IgG        | 35.7            | 89.4            | 73.5            | 62.8            | 3.37            | 0.72            |
| ASCA IgA        | 28.7            | 84.6            | 60.5            | 59.1            | 1.86            | 0.84            |
| ASCA either     | 45.0            | 79.8            | 64.7            | 63.8            | 2.23            | 0.69            |
| ASCA both       | 19.3            | 94.2            | 73.3            | 58.7            | 3.35            | 0.86            |
| GP2 IgG         | 42.7            | 86.1            | 71.6            | 64.6            | 3.06            | 0.67            |
| GP2 IgA         | 33.9            | 88.9            | 71.6            | 62.1            | 3.07            | 0.74            |
| GP2 either      | 49.7            | 79.8            | 66.9            | 65.9            | 2.46            | 0.63            |
| GP2 both        | 26.9            | 95.2            | 82.1            | 61.3            | 5.60            | 0.77            |
| ASCA+/ANCA-     | 41.5            | 92.8            | 82.6            | 65.9            | 5.76            | 0.86            |
| GP2+/ANCA-      | 43.3            | 90.9            | 79.6            | 66.1            | 4.74            | 0.62            |
| GP2+/ASCA-      | 29.8            | 93.8            | 79.7            | 61.9            | 4.77            | 0.75            |
| GP2+/ASCA+       | 28.1            | 97.1            | 88.9            | 62.2            | 9.73            | 0.74            |

| UC vs. CD       | Sensitivity (%) | Specificity (%) | PPV (%)         | NPV (%)         | LR+             | LR-             |
| ANCA IgG        | 55.8            | 88.3            | 85.3            | 37.9            | 4.77            | 0.50            |
| ANCA IgA        | 29.8            | 93.6            | 84.9            | 47.7            | 4.63            | 0.75            |
| ANCA either     | 60.6            | 87.1            | 85.1            | 35.5            | 4.71            | 0.45            |
| ANCA both       | 25.0            | 94.7            | 85.2            | 49.1            | 4.75            | 0.79            |
| ANCA+/ASCA-     | 47.6            | 90.6            | 86.1            | 41.3            | 5.09            | 0.58            |
| ANCA+/GP2-      | 49.5            | 93.6            | 90.4            | 39.6            | 7.70            | 0.54            |
| ANCA+/GP2+/ASCA- | 39.9          | 95.3            | 91.2            | 43.4            | 8.53            | 0.63            |

| CD vs. intestinal BD | Sensitivity (%) | Specificity (%) | PPV (%)         | NPV (%)         | LR+             | LR-             |
| ASCA IgG        | 35.7            | 80.0            | 82.4            | 32.1            | 1.78            | 0.80            |
| ASCA IgA        | 28.7            | 75.4            | 75.4            | 28.7            | 1.16            | 0.80            |
| ASCA either     | 45.0            | 67.7            | 78.6            | 31.9            | 1.39            | 0.51            |
| ASCA both       | 19.3            | 87.7            | 80.5            | 29.2            | 1.57            | 0.92            |
| GP2 IgG         | 42.7            | 84.6            | 88.0            | 35.9            | 2.77            | 0.68            |
| GP2 IgA         | 33.9            | 86.2            | 86.6            | 33.1            | 2.45            | 0.77            |
| GP2 either      | 49.7            | 75.4            | 84.2            | 36.3            | 2.02            | 0.67            |
| GP2 both        | 26.9            | 95.4            | 93.9            | 33.2            | 5.83            | 0.77            |

| CD vs. ITB      | Sensitivity (%) | Specificity (%) | PPV (%)         | NPV (%)         | LR+             | LR-             |
| ASCA IgG        | 35.7            | 84.6            | 91.0            | 23.1            | 2.32            | 0.76            |
| ASCA IgA        | 28.7            | 79.5            | 86.0            | 20.3            | 1.40            | 0.90            |
| ASCA either     | 45.0            | 74.4            | 88.5            | 23.6            | 1.76            | 0.74            |
| ASCA both       | 19.3            | 89.7            | 89.2            | 20.2            | 1.88            | 0.90            |
| GP2 IgG         | 42.7            | 84.6            | 92.4            | 25.2            | 2.77            | 0.68            |
| GP2 IgA         | 33.9            | 87.2            | 92.1            | 23.1            | 2.65            | 0.76            |
| GP2 either      | 49.7            | 74.4            | 89.5            | 25.2            | 1.94            | 0.68            |
| GP2 both        | 26.9            | 97.4            | 97.9            | 23.3            | 10.49           | 0.75            |

*aASCA+, ASCA either; GP2+, GP2 either; ANCA+, ANCA either; ASCA-, both ASCA IgA and ASCA IgG negative; GP2-, both GP2 IgA and GP2 IgG negative; ANCA-, both ANCA IgA and ANCA IgG negative, CD, Crohn's disease; UC, ulcerative colitis; BD, Behçet's disease; ITB, intestinal tuberculosis; ASCA, anti-Saccharomyces cerevisiae antibody; ANCA, anti-neutrophil cytoplasmic antibodies; GP2, anti-zymogen granule glycoprotein 2 antibodies; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio; N.A., not applicable.

Figure 2 Venn diagram describing the relationships between serological markers (aGP2 and ASCA) in CD cohort (n = 171).
autoantibodies. Additionally, IgG aGP2, IgA/G aGP2, IgA/G ASCA, ASCA+/aGP2- were negatively correlated with A3, while ASCA-/aGP2- were positively correlated with A3, suggesting that the CD patients diagnosed after 40 years old were less likely to have ASCA or aGP2.

Furthermore, a significant negative association was found between the combination of ASCA+/aGP2- and dermatologic involvement, indicating that patients with ASCA+/aGP2- were less likely to have dermatologic involvement (\(P = 0.030\)). In addition, patients with IgA+G ASCA were more likely to display steroids resistance (\(P = 0.047\)).

As CD patients displayed wide variations in terms of age at diagnosis, disease duration and other factors, a logistic regression model was utilized to assess how those confounding factors affected the diagnostic characteristics of aGP2 (Table 6). Only IgG aGP2 remained an independent risk factor for the presence of B3 along with the confounding factors gender, indicating that males with IgG aGP2 positivity had a higher risk for B3. Further, IgG aGP2 was a predictor for severe disease. The negative association for IgG ASCA with regard to the latter outcome did not reach significance (\(P > 0.05\)). Other confounding factors were age (older patients) and short disease duration. Further, IgG ASCA was a significant predictor for the response to steroids in patients receiving 5-ASA without stenosing behavior and shorter duration of disease. Patients with dermatologic extraintestinal manifestations having been prescribed no anti-TNF treatment revealed IgA ASCA as a predictor.

**DISCUSSION**

In this study, we evaluated the diagnostic potential of aGP2 in a large, well-defined Chinese cohort with a special focus on the role of aGP2 in distinguishing CD from intestinal BD and ITB. Altogether, aGP2 displayed a better discriminatory capability over ASCA in differentiating CD from UC, CD from intestinal BD, and CD from ITB. In addition, aGP2 was significantly associated with ileal disease. Further, aGP2 was linked to a higher risk for developing a more aggressive disease phenotype (B3), suggesting that the presence of aGP2 may predict individuals who are particularly susceptible to the development of complicated CD behavior. Degenhardt et al. reported the association of aGP2 with the need for surgical intervention which supports our findings. The identification of patients at risk would allow an early or more aggressive therapeutic intervention. Taken together, our data suggest that the inclusion of both IgA and IgG aGP2 testing into the routine screen test panels may enhance the overall performance of serological tests for diagnosis of CD, especially in countries with high prevalence of intestinal BD.
### Table 5: Clinical relevance of aGP2 and ASCA with disease characteristics in patients with Crohn’s disease

| Parameters | IgG aGP2 | IgA aGP2 | aGP2 either | IgG ASCA | IgA ASCA | ASCA either | ASCA both | ASCA+ aGP2 | ASCA- aGP2 | ASCA+ aGP2 | ASCA- aGP2 | ASCA+ aGP2 | ASCA- aGP2 |
|------------|---------|---------|-------------|---------|---------|-------------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|
| Age at diagnosis | 0.039 | 0.045 (0.2, 0.8) | 0.011 0.4 | 0.045 0.5 | 0.045 (0.2, 1.0) | 0.035 0.4 | 0.041 0.4 | 0.035 (0.1, 0.9) | 0.014 0.4 | 0.019 2.4 | 0.003 0.2 | 0.014 0.4 |
| Hormatologic resistance | 0.003 2.4 | 0.018 0.4 | (0.2, 0.9) | 0.033 0.5 | 0.022 2.4 | 0.022 (1.1, 5.2) | 0.011 2.9 | 0.011 (1.2, 7.0) | 0.011 2.0 | 0.018 0.4 | (0.2, 0.9) | 0.011 2.0 |
| Steroids resistance | 0.003 2.4 | 0.022 2.4 | (1.1, 5.2) | 0.026 2.2 | 0.026 2.2 | 0.026 (1.1, 4.2) | 0.022 2.4 | 0.022 (1.1, 5.3) | 0.022 2.0 | 0.026 2.4 | (1.1, 5.3) | 0.026 2.0 |
| Dermatologic resistance | 0.009 2.4 | 0.015 2.4 | (1.2, 4.1) | 0.003 2.6 | 0.003 2.6 | 0.003 (1.2, 4.8) | 0.012 2.4 | 0.012 (1.2, 4.8) | 0.012 2.0 | 0.015 2.0 | (1.2, 4.0) | 0.015 2.0 |

Clinical relevance of the markers with disease characteristics in patients with Crohn’s disease expressed as P-Values and OR (95% confidence interval, CI). Only significant relevance for a given parameter is shown. Positive associations are indicated in bold and negative associations in italic (P-Values, odds ratio and range). A represents age at diagnosis (A3, above 40 years), L represents the location of disease (L1, ileal; L3, ileocolonic), and B represents disease behavior (B2, stricturing; B3, penetrating; p, perianal disease modifier) based on the Montreal Classification. ASCA, anti-Saccharomyces cerevisiae antibody; aGP2, anti-zymogen granule glycoprotein 2 antibodies.

**aASCA +, ASCA either; aGP2 +, aGP2 either; ASCA - both ASCA IgA and ASCA IgG negative; aGP2 - both aGP2 IgA and aGP2 IgG negative.**

**b** The correlation between age at diagnosis with the antibodies was assessed with Spearman’s rank correlation coefficient.
Severe disease correlated with stricturing phenotype (B2)20,39,40. Interestingly, previous studies reported that aGP2 were associated with a more aggressive disease phenotype (B3), suggesting that the presence of aGP2 may identify patients at risk of developing complicated CD disease onset. Taken together, our data help to delineate a more complicated penetrating disease behavior and early disease onset. A recent study also found a significant association between aGP2 and the penetrating phenotype (B3)41. The discrepancy might be due to differences in sample size and ethnic/geographic backgrounds. Further, IgA aGP2 was identified as a predictor for the occurrence of B3 in patients with CD. Interestingly, previous studies reported that aGP2 were correlated with stricturing phenotype (B2)20,39,40. However, a recent study also found a significant association between aGP2 and the penetrating phenotype (B3)41. The discrepancy might be due to differences in sample size and ethnic/geographic backgrounds. Further, IgG aGP2 was identified as a predictor for severe CD with several confounding factors supporting the association of IgG aGP2 with a severely complicated CD phenotype as reviewed recently.18

A recent study showed that IgA aGP2 were present in approximately 50% patients with primary sclerosing cholangitis (PSC).29 Of note, IgA aGP2 displayed a similar prevalence in PSC patients without concomitant IBD compared with PSC patients with IBD. This suggests that IgA aGP2 may be also associated with the inflammation in bile ducts apart from the one in small intestine.

Interestingly, we also found that aGP2 were negatively associated with age at diagnosis, which was consistent with our studies.20,40 In particular, IgG aGP2 and IgA/G aGP2 were negatively correlated with A3, indicating that the presence of aGP2 might be associated with an early disease onset.

It has been shown that GP2 displays anti-inflammatory effects by decreasing proliferation, apoptosis, and activation of intestinal epithelial cells and mucosal and peripheral T-cell, as well as inhibiting pro-inflammatory chemokine CXCL8 and upregulating anti-inflammatory cytokine TGF-β1.42 Thus, IgG aGP2 may block the suppressive effect of GP2, thereby promoting intestinal inflammation. In addition, IgA aGP2 may bridge FimH-positive pathogen bound-GP2 with M cell surface GP2, resulting in elevated transcytosis of GP2-covered pathogens.43 Our finding that IgG and IgA aGP2 as well as combinations thereof were correlated with a more aggressive disease phenotype supports the hypothesis that aGP2 indeed can play a pathogenic role in exacerbation or perpetuation of CD inflammation.

In conclusion, our data suggest that aGP2 displayed a better discriminatory performance over ASCA in differentiating CD from UC, CD from intestinal BD, and CD from ITB. The presence of aGP2 could identify CD patients with ileal location, a more complicated penetrating disease behavior and early disease onset. Taken together, our data help to delineate further the clinical utility of aGP2 in the diagnosis of CD, especially when it comes to distinguishing CD from intestinal BD or ITB.

CONFLICT OF INTEREST

Guarantor of the Article: Shulan Zhang, MD and Yongzhe Li, MD.

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Table 6 Logistic regression analyses for autoantibodies in patients with Crohn’s disease

| Coefficient | Std.Error | Odds ratio | 95% CI | P Value |
|-------------|-----------|------------|--------|---------|
| B3 | | | | |
| IgG aGP2 | 0.005 | 0.002 | 1.005 | 1.002, 1.009 | 0.0044 |
| Gender | 0.978 | 0.430 | 2.660 | 1.144, 6.184 | 0.0230 |
| Severe disease | | | | |
| IgG aGP2 | 0.011 | 0.003 | 1.012 | 1.006, 1.018 | 0.0002 |
| IgG ASCA | -0.035 | 0.020 | 0.965 | 0.928, 1.005 | 0.0822 |
| Age | 0.053 | 0.022 | 1.055 | 1.011, 1.100 | 0.0134 |
| Duration | -0.126 | 0.061 | 0.881 | 0.781, 0.994 | 0.0397 |
| Steroids response | | | | |
| IgG ASCA | 0.015 | 0.008 | 1.016 | 1.001, 1.031 | 0.0426 |
| 5-ASA | 1.168 | 0.364 | 3.215 | 1.574, 6.566 | 0.0013 |
| B2 | -0.592 | 0.355 | 0.553 | 0.276, 1.109 | 0.0953 |
| Duration | -0.088 | 0.034 | 0.915 | 0.857, 0.978 | 0.0083 |
| Dermatologic | | | | |
| IgA ASCA | 0.008 | 0.004 | 1.008 | 1.000, 1.016 | 0.0461 |
| Anti-TNF | -1.307 | 0.517 | 0.271 | 0.098, 0.746 | 0.0116 |

B represents disease behavior (B2, stricturing; B3, penetrating; p, perianal disease modifier) based on the Montreal Classification. 5-ASA, therapy with 5-aminosalicylic acid; aGP2, anti-zymogen granule glycoprotein 2 antibodies; ASCA, anti-Saccharomyces cerevisiae antibody; Anti-TNF, therapy with anti-TNF; Duration, disease duration; Location of disease (L, ileal); age, age at diagnosis; Geographic Background, geographic background; Significant relationships between one dichotomous dependent variable (various clinical outcomes or treatment variants) and one or more independent variables including aGP2 and ASCA, gender, age, disease duration, surgery are shown (P<0.003 respectively). (only significant correlations are shown).
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Specific author contributions: SZ and JL designed and performed the study, and drafted the manuscript. ZW and JL performed the study. DRO designed the study, and contributed by critical revision of the manuscript. PS and Dre critically revised the manuscript and participated in the statistical evaluation of the data. JQ and YL designed the study and drafted the manuscript. All authors were responsible for data analysis and interpretation.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE
✓ The discriminatory capability of ASCA in diagnosing patients with CD is far from satisfactory, indicating a clear need for other biomarkers with highly sensitive and specific diagnostic power.
✓ Antibodies to glycoprotein 2 (aGP2) have demonstrated promising potential in the diagnosis of CD. However, no study has assessed the clinical relevance of aGP2 in differentiating CD from intestinal Behçet's disease (BD) or CD from intestinal tuberculosis (ITB).

WHAT IS NEW HERE
✓ IgG aGP2 and IgA aGP2 demonstrated significantly higher AUC than IgG ASCA and IgA ASCA in distinguishing CD from intestinal BD or CD from ITB.
✓ aGP2 displayed a better discriminatory capability over ASCA in differentiating CD from UC, CD from intestinal BD, and CD from ITB.
✓ The presence of aGP2 could identify CD patients with ileal location, a more complicated penetrating disease behavior and early disease onset.

TRANSLATIONAL IMPACT
✓ Our findings demonstrated that aGP2 displayed a better discriminatory capability over ASCA in differentiating CD from UC, CD from intestinal BD, and CD from ITB.
✓ Our data suggest that the inclusion of both IgA and IgG aGP2 testing into the routine screen test panels may enhance the overall performance of serological tests for diagnosis of CD, especially in countries with high prevalence of intestinal BD or ITB.

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