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Chapter 6

The Utility of Serological Markers in Inflammatory Bowel Diseases: Gadget or Magic?

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Abstract

The panel of serologic markers for inflammatory bowel diseases (IBD) is rapidly expanding. Anti–*Saccharomyces cerevisiae* antibodies (ASCA) and atypical perinuclear antineutrophil cytoplasmic antibodies (P-ANCA) remain the most widely investigated; however there are methodological difficulties and no clear guidelines for immunofluorescence detection and interpretation of ANCA patterns in IBD hampering the diagnostic potential of the test. Increasing amount of experimental data is available on newly discovered antibodies directed against various microbial proteins and carbohydrates. Such antibodies include anti-OmpC (outer membrane porin C), anti-*Pseudomonas fluorescens* (anti-I2), anti-flagellin antibody CBir1 and antiglycan antibodies (anti-laminaribioside carbohydrate antibody [ALCA], anti-chitobioside carbohydrate antibody [ACCA], anti-mannobioside carbohydrate antibody [AMCA]).

The pathogenic significance of these antibodies has not been established and it remains unclear whether they arise as a result of tissue damage, increased permeability or the mucosal immune perturbation seen in Crohn's disease. Reactivity to microbial components was associated with NOD2/CARD15 genotype. Moreover, positive

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correlation was found between the number of mutations and the prevalence of antimicrobial antibodies (gene dosage effect), further supporting the role of altered microbial sensing in the pathogenesis of Crohn's disease.

The role of the assessment of various antibodies in the current IBD diagnostic algorithm is often questionable due to their limited sensitivity. In contrast, the association of serologic markers with disease behaviour and phenotype is becoming increasingly well-established. An increasing number of observations confirm that patients with Crohn's disease expressing multiple serologic markers at high titers are more likely to have complicated small bowel disease (e.g. stricture and/or perforation) and are at higher risk for surgery than those without, or with low titer of antibodies (serology dosage effect). Creating homogenous disease sub-groups based on serologic response may help develop more standardized therapeutic approaches and may help in a better understanding of the pathomechanism of inflammatory bowel diseases. Further prospective clinical studies are needed to establish the clinical role of serologic tests in IBD.

Keywords: serologic markers, inflammatory bowel disease, ulcerative colitis, Crohn's disease, indeterminate colitis.

Introduction

Serologic response to various microbial and autoantigens can develop in inflammatory bowel diseases (IBD). In addition to the well-established atypical perinuclear antineutrophil cytoplasmic antibodies (atypical P-ANCA) and anti–*Saccharomyces cerevisiae* mannan antibodies (ASCA), a number of the new antibodies have recently been discovered and data on their clinical significance has been rapidly increasing.

The usefulness of different antibodies in Crohn's disease (CD) and ulcerative colitis (UC) as diagnostic markers, follow-up parameters, or as subclinical markers in affected families has been actively investigated. Another field of interest is the association of the serologic markers with the disease phenotype, disease course, and treatment stratification. The role of the antibodies in disease pathophysiology remains to be fully elucidated.

In this review we discuss current understanding on the clinical importance of various established and newly recognized serologic markers in IBD.

Serologic Panel for Inflammatory Bowel Disease (IBD)

Anti-Neutrophil Cytoplasmic Antibody (ANCA)

The classic ANCA tests are used to diagnose and monitor the inflammatory activity in primary small vessel vasculitides. On the basis of an international consensus statement, ANCA testing is performed with serum samples by indirect immunofluorescence (IIF) on normal peripheral blood neutrophils. Two basic ANCA patterns are detectable: the cytoplasmic (C-ANCA) and the perinuclear (P-ANCA). The C-ANCA pattern appears as a

granular, diffuse cytoplasmic fluorescence, often with accentuated fluorescence around the nuclear lobes. Typical P-ANCA reactivity results in homogeneous rim-like staining of the perinuclear cytoplasm. ANCA positive serum samples and also those with any other cytoplasmic fluorescence or an antinuclear antibody (ANA) that results in homogeneous or peripheral nuclear fluorescence should be tested in enzyme-linked immunosorbent assays (ELISA) for proteinase 3 (PR3) and myeloperoxidase (MPO) antibodies, because these are the most common of targets of C-ANCA and P-ANCA antibodies respectively (*minimum recommendation of consensus group*). Optimally, ELISAs should be performed on all serum samples, since IIF alone detects only 90% to 95% of all ANCA positive serum samples in patients [1]. A third ANCA pattern of clinical importance is the so-called atypical P-ANCA staining. It has been suggested that since the target antigens of atypical P-ANCA are nuclear rather than cytoplasmic, this pattern would be more properly named ANNA (anti-neutrophil nuclear antigen). Until the target of atypical P-ANCA reactivity is identified however, it is likely that the atypical nomenclature will remain in common use. Atypical P-ANCA is recognized as a broad inhomogeneous rim-like staining of the nuclear periphery often with multiple intranuclear foci [2]. The antigen specificity of these atypical ANCAs are different from the classic C-and P-ANCAs, being localized in the nuclear periphery, in contrast to the cytoplasmic location of the classic C- and P-ANCAs. Atypical P-ANCA are most commonly seen in patients with IBD, especially UC, and some autoimmune liver diseases such as autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC).

Atypical P-ANCA is present in the sera of 40% to 80% of patients with UC [3,4] and to a lesser extent in CD (5-25%) [5]. The prevalence of the antibody is also high in patients with PSC (88%) [6] and AIH type I (81%) [7], but is detected in only 1-3% of healthy control subjects. Some sera with atypical ANCA reactivity are positive for antibodies to elastase, lactoferrin, cathepsin G, lysosyme or bactericidal permeability-increasing protein [BPI]), but since they are only detected in a few atypical P-ANCA positive sera, these antigens do not appear to be the primary targets of atypical P-ANCA reactivity [8]. The target antigen(s) of atypical P-ANCA have not been definitively identified. What is in agreement is that target antigen(s) are associated with inner side of the neutrophil nuclear membrane. A 50-kilodalton myeloid-specific protein has been identified by *Terjung* and appears to be the best current candidate as the primary target of atypical P-ANCA[2].Histone H1, which binds to the DNA linking nucleosomes, has been suggested as a target antigen of atypical P-ANCA [9]. However, histone H1 is found in all cells with nucleus and is not specific to neutrophils. There has been little independent support of this idea. More recently, the proposed anti-α-enolase antibodies were present in a substantial proportion of patients with IBD but also in various inflammatory/autoimmune disorders, and non-IBD gastrointestinal controls. And no association was found between the presence of anti-α-enolase antibodies and the P-ANCA status of patient with IBD. Absorption with α-enolase before IIF did not eliminate the P-ANCA staining pattern, which is consistent with the idea that α-enolase is not the target antigen of P-ANCA [10]. Since the exact target antigen(s) of atypical P-ANCA has not been identified, there are currently no sensitive and specific solid-phase assays available to screen for these antibodies. The IIF is the only widespread method to detect the antibodies; however it is technically demanding, subjective, and requires experienced observers for good interpretation. Unlike the Consensus Recommendations for the vasculitis-associated ANCAs,

there are no clear guidelines for immunofluorescence detection and interpretation of atypical P-ANCA patterns. A reproducible and specific method was described by *Terjung et al*, who used the combination of ethanol-fixed and formalin-fixed human neutrophil substrates do discriminate between P-ANCA and atypical P-ANCA. Using the cross-linking fixative formalin, typical P-ANCA diffusely labeled the cytoplasm, that is, converted to cytoplasmic ANCA pattern. In contrast, sera containing atypical P-ANCA produced a fine 'perinuclear' labeling with multiple intranuclear fluorescent foci. This pattern, however, was obvious only with confocal laser scanning microscopy [11]. When lower resolution planar indirect immunofluorescence microscopy is used, this labeling is difficult to detect, and therefore the fluorescence is usually considered negative[12, 13]. However, simultaneous reactivity of atypical P-ANCA on formaldehyde- and ethanol-fixed neutrophils and the feasibility of the new microscopic criteria have not been systematically studied in IBD. Most studies dealing with serological evaluation of IBD use ethanol-fixed neutrophils only, rendering them biased by MPO-ANCA, antinuclear antibodies (ANA) and other, non-IBD associated antibodies (anti-lamin, anti-Golgi, anti-actin, etc. [14]).

ANCA systems that replace formalin-fixed neutrophils with an enzyme (DNase I) digestion step during IIF were developed by *Targan* and colleagues [15], but rarely performed outside the group. Instead of the term "atypical", they rather use "DNase-sensitive" (i.e., not detectable on DNase-treated neutrophils) P-ANCA [16,17].

The poor agreement between various ANCA assays and different observers is well documented [18,19]. The remarkable differences are likely attributable to several distinctive parameters of the various manufacturer kits (differences in cell preparation affecting background fluorescence, fixation methodologies, buffers and conjugates). Discrepancies are further increased by different microscopes and the observers' various experience level in inter-observer studies.

In a recent study[8], in a group of 204 patients with IBD we attempted to differentiate among C-ANCA, P-ANCA and atypical P-ANCA based on the occurrence of the patterns on ethanol- and formalin-fixed slides and using predetermined IIF criteria. The most often detected ANCA pattern was atypical P-ANCA both in UC and CD patients. However, we found a significant variation in the occurrence of atypical P-ANCA in the sera of patients with UC (9.4-39.6%) and CD (9.3-16.7%) among different, commercially available ANCA assays (INOVA, IMMCO, Immunoconcepts, Euroimmun). All possible pair-wise comparisons demonstrated a statistically significant lack of agreement between the ANCA assays (κ: 0.14-0.34). Similar results were published in two earlier studies. *Joossens et al*[18] assayed the presence of ANCAs by four different, commercially available substrates (Bio-Rad, The Binding Site, Immunoconcepts, and INOVA) in 50 UC patients. The prevalence of atypical P-ANCA varied between 16% and 62% using both ethanol- and formalin-fixed human neutrophil substrates, with κ values < 0.2 indicating poor agreement. In the paper of *Sandborn et al*[19], the sensitivity for P-ANCA detection in 162 IBD patients varied between 0% and 63% in different laboratories (Prometheus, Oxford, Wuerzburg, Mayo, and Smith Kline Beecham). One of the assays in this study used the DNase digestion method, while the others only used ethanol-fixed slides.

In the inter-observer study, we found better concordance for atypical P-ANCA (κ=0.44), suggesting that the differences between IIF ANCA assays from various sources contributed more to the discrepancies than the type of the microscope or the observers' experience. These values are in accordance with our previously published observation on the prevalence of P-ANCA in IBD [20].

The overall specificity of the atypical P-ANCA is 84-95%, sensitivity is 48-63%, positive predictive value (PPV) is 69%, and negative predictive value (NPV) is 89%[5].

ANCA production in UC appears to be genetically conditioned by promoter polymorphism of both anti- and proinflammatory cytokine molecules. The high producer TNF-α genotype clearly correlated with ANCA positivity as well as the low producer IL-10 genotype. Furthermore, combination of both genotypes had a greater influence on ANCA positivity than each individual genotype. One possible explanation would be that the incremented local synthesis of TNF-α that cannot be counterbalanced by a low production of IL-10 in patients may create a microenvironment in which autoimmune inflammatory response would tend to perpetuate [21].

Anti–*Saccharomyces Cerevisiae* Antibodies (ASCA)

ASCA are antibodies directed primarily against a 200 kDa- phosphopeptidomannan cell wall component of the common baker's or brewer's yeast *Saccharomyces (S.) cerevisiae* [22]. ASCA reactivity could be a result of cross-reacting antibodies to antigens found in a non-yeast organism and has not yet been identified [23,24]. Mannose is not only found in yeast but also in mycobacteria and other microorganisms [25]. Both IgA and IgG antibodies are formed. Separate and polyvalent ELISA configurations are available for ASCA IgG and IgA detection. ASCA are more frequently found in CD patients (50-80%) compared to patients with UC (2-14%) and to normal healthy subjects (1-7%) [26,27]. Approximately two-thirds of the CD patients with ASCA IgG are also positive for ASCA IgA, but from 0 to 19% of the patients have only ASCA IgA antibodies. This suggests that both ASCA IgG and IgA antibodies should be measured. In CD, up to 90% specificity has been reported in specimens positive for both ASCA IgG and IgA antibodies, especially when the magnitude of both the IgG and IgA ASCA antibodies is high [28]. Sensitivity of ASCA testing ranges from 41- 76%, PPV 88% and NPV 68% [29]. More recently a large number of IBD and control sera, previously tested for ASCA, were used for evaluating a new antisynthetic oligomannoses antibody (AΣMA) test by *Vandewalle-El Khoury et* al. AΣMA revealed the heterogeneity of the antioligomannose antibody response in CD patients and increased the sensitivity of CD diagnosis when combined with ASCA. Of the ASCA negative CD patients 25% were positive for AΣMA [30].

ASCA IgG and IgA levels in CD patients are highly variable[28]. The prevalence of ASCA is much higher in cases of sporadic CD and in families with only CD (63%) compared to families with both CD and UC (33%). The familial trait to ASCA is obvious, but it is questionable whether this is due to the genetic background or environmental agents effect in the childhood predisposing to the disease susceptibility.

A comparative study revealed a wide range in sensitivities and specificities among four assays, mainly as a consequence of the cut-off values chosen. Sensitivity was inversely related to specificity and PPV. Results correlated well overall and the different ROC curves showed good agreement [31] We also compare the clinical accuracy of gASCA IgG and conventional ASCA IgG in a total of 652 IBD patients. The performances of the two IgG test were similar. The weighted Kappa coefficients (κ) suggested good concordance between the two assays (κ: 0.67, $p<0.0001$). gASCA and ASCA IgG positivity were observed in 50.4% and 50.6% CD patients, respectively. The combined positivity rate was 58.9%. Both tests identified 83.5% of the same patients; however, 8.4% and 8.3% of all patients were identified only by one of the tests. This suggests that while the antigens used in the two assays are similar, they also contain different non-overlapping epitopes [32].

Newly Discovered Serologic Markers

Anti-OmpC antibody is directed against the outer membrane porin C transport protein of the *Eschericia coli*. The detection of the IgA antibody is done with ELISA. Anti-OmpC has been reported in 24-55% of CD patients [33,34]. The prevalence of anti-OmpC was insignificant in UC patients and in healthy subjects (5-11% and 5%, respectively). However, others reported higher occurrence of this antibody both in UC and healthy controls using another commercially available OMP ELISA test [20, 35]. Anti-OmpC maybe of value to aid diagnosis of ASCA negative CD patients. The prevalence of anti-OmpC among ASCA negative patients is 5-15%.

A fragment of bacterial DNA (I2), a homolog of the tetR bacterial transcriptional factor family, has been identified from lamina propria mononuclear cells in active CD and shown to be associated to *Pseudomonas fluorescens* [36,37]. IgA anti-I2 antibody has been detected by ELISA in IBD patients with a seroprevalance of 54% in CD and 10% in UC. Anti-I2 antibody was also found in patients with other inflammatory enteritis (19%) and also in healthy subjects (4%)[5].

Serologic expression cloning was used by *Lodes et al*. to identify commensal bacterial proteins in colitic mice. The dominant antigens were found to be flagellins. Strong B-cell and CD4+ T-cell responses were observed against one of these flagellins (*anti-CBir1).* Colitis was induced when the T-cell line specific for CBir1 was transferred into naive severe combined immunodeficient mice. Approximately 50% of patients with CD have IgG serum reactivity to CBir1 versus 6% of UC patients and 8% of healthy subjects. CBir1 is the first bacterial antigen to induce colitis in animal models of IBD and also leads to a pathological immune response in IBD patients [38]. Among the population of CD patients positive for atypical P-ANCA, but who do not react to other known antigens, 40-44% are positive for anti-CBir1 whereas the antibody has only been found in only 4% of atypical P-ANCA positive UC patients. Serum responses to CBir1 maybe of help in differentiation between atypical P-ANCA positive CD and UC patients independently of ASCA [39,40].

Anti-pancreatic autoantibodies (**PAB**) are directed against the exocrine pancreatic tissue [41]. The exact target antigen(s) however, have not yet been identified. The detection of PAB is done by IIF on human or primate pancreas substrate and the reported prevalence in small

scale studies was 27–39% in CD patients compared to 0-5% of UC patients and 0-8% of healthy subjects [42,43,44,45]. Based on these results PABs have been suggested to be highly specific for CD. However, a more recent study from Belgium found a much higher prevalence (22.2%) of PAB in UC [46]. The occurrence of PAB was similar to the above mentioned data in our IBD cohort which is the largest known patient group so far (CD= 579, UC=110). The autoantibody was specific for CD (92-95%), while sensitivity alone was only 38-41%, albeit the same PAB prevalence was found in celiac disease at the diagnosis than in UC (23.3% vs. 22.7%) (unpublished results). The relevance of PAB in the pathogenesis of CD is unclear and whether the presence of PAB identifies a CD subgroup also remains to be determined [47].

Earlier studies showed great variation in the frequency of *goblet cell antibodies* in IBD (0%-40%)[45, 48,49] and found these autoantibodies as specific markers to UC. In these studies, tissue samples from other species, for example monkey or intestinal goblet cell culture were used. More recently *Ardesjo et al* [50] reported that 84% of IBD patients, both UC and CD, have antibodies against goblet cells in the human appendix substrate. The specificity of goblet cell antibodies in this study was high (92%) for distinguish IBD patients from healthy controls but lower (50-60%) when comparing IBD to the control gastrointestinal inflammatory diseases. However the staining pattern was different and its intensity was weaker in celiac disease and infectious gastroenteritis as compared to IBD patients suggesting different goblet cell antigens. The results of this study propose that immunoreactivity against goblet cells may be of importance in the pathogenesis of IBD however identification of goblet cell antigens is mandatory.

Patients with CD express antibodies to cell wall carbohydrate epitopes found in different micro-organisms, immune cells, erythrocytes, and tissue matrices. Using a glycan array (GlycoChip) and ELISA, in addition to antibodies against mannan (anti-covalently attached mannan from *Saccharomyces cerevisiae* IgG antibodies or gASCA) other **anti**-*glycan antibodies* have been found including anti-mannobioside [(Man(α1,3)Man(α) carbohydrate IgG antibody or AMCA], antibodies against laminaribioside [anti-laminaribioside (Glc(β1,3)Glc(β)) carbohydrate IgG antibodies or ALCA, a building block of laminarin, which may be found in the cell walls of saprophytic and pathogenic fungi and yeast] and chitobioside [anti-chitobioside (GlcNAc(β1,4)GlcNAc(β)) carbohydrate IgA antibodies or ACCA, a component of chitin, a major element of the insect cuticle and cell walls of infectious pathogens such as bacteria and yeast] [51].

The positivity rate was 15-18% for ALCA, 11-21% for ACCA and 12-28% for AMCA in two large, independent cohort from Western [35] and Eastern-Europe[32]. Interestingly, the positivity rate was somewhat higher in a study from Israel in both the initial and the validated CD cohorts [52]. However, we must note that AMCA was not measured and the total patient number in the latter study was relatively small, which may at least partly contribute to the heterogeneity observed among these studies. In generally, these anti-glycan markers were highly specific for CD, but their sensitivities were poor.

Comparing all 4 anti-glycan antibodies' performance to the results of the combined IgG or IgA ASCA, we found that both panels identified 59.4% of all CD patients. These data suggests that there is no advantage of using anti-glycan markers instead of traditional ASCA IgG and A, not to mention the additional cost. Nonetheless, the two panels identified the

same patients in only approximately 80% of the cases, while 10.8% of all patients were identified only by the two conventional ASCA tests and another 10.8% by anti-glycans. Either of the two panels was positive in 70.2% of CD patients.

The Diagnostic Value of the Serologic Markers in IBD

The role of atypical P-ANCA and ASCA as diagnostic markers for IBD appears to be limited because of their moderate sensitivity and presence in other conditions. It must be emphasized that neither ASCA, nor atypical P-ANCA negativity rules out IBD. Similarly, the presence of these antibodies does not confirm the diagnosis of IBD. Atypical P-ANCA can also be observed in other colitis, e.g. collagenous or eosinophilc colitis and in various autoimmune liver diseases such as AIH and PSC [53,54] ASCA has been found in autoimmune hepatitis (20%) and gastrointestinal disorders such as celiac disease [55]. The frequency of ASCA at diagnosis of the celiac disease varied from 27% to 59% in the different studies owing to the differences in the number and the age of the patients as well as the commercial assays used for antibody detection [56,57,58,59,60]. The higher frequency of ASCA positivity has also been reported in adult celiac cohorts compared to the pediatric ones supposedly due to the longer gluten exposition and therefore bearing more lingering gut mucosal damage. In the majority of patients, ASCAs disappeared during sufficiently long-lasting gluten free-diet (GFD). However, in children the disappearance of ASCA positivity has been more pronounced attributed to the well-known fact that gut permeability normalizes much better in children than in adults. In addition to ASCA, our group also found anti-glycan and anti-OMP antibody frequencies significantly higher in celiac patients at diagnosis. Positivity for these antibodies were completely lost after adherent long-standing GFD[61].

The combination of atypical P-ANCA and ASCA however, may be of help in patients in whom distinction between CD and UC is not obvious with the classic diagnostic tools (patient history, radiologic examination, endoscopy and biopsy). The $ASCA^+$/ atypical P-$ANCA^-$ serologic pattern is mainly characteristic of CD, while the $ASCA^-$/ atypical P-$ANCA^+$ is characteristic of UC. Several independent studies found that these combinations had sensitivities of from 30% to 64%, specificity more than 90%, and PPV from 77% to 96% [15, 26,27,62,63].

The difficulty in the clinical differentiation between CD and UC, however, lies in distinguishing isolated colonic CD from UC, as ileal and proximal small-bowel involvement occurs only in CD. ASCA IgA and IgG double positivity (PPV for CD: 83%), pANCA+/ASCA- (PPV for UC: 80%) and pANCA-/ASCA+ pattern (82%) were associated with reasonable positive predictive values, nonetheless the sensitivity was low. Including Omp in the analysis had no additional value and resulted in even lower sensitivity and specificity values [20]. ASCA in combination with PAB autoantibodies also increases the sensitivity to detect isolated colonic CD.

Serologic evaluation may be of help in patients with *indeterminate colitis (IC)* to increase the diagnostic accuracy. Ninety-seven patients with IC were enrolled, analyzed for atypical P-ANCA and ASCA, and followed up prospectively in a multicentre study of *Joosens et*

al.[64]. After the 1-year follow-up, a definitive diagnosis was reached in 31 of 97 patients (37%). In IC patients, $ASCA^+$/ atypical $P\text{-}ANCA^-$ results correlated with CD in 80%, whereas $ASCA^-$/ atypical $P\text{-}ANCA^+$ correlated with UC in 63%. The remaining $ASCA^-$/ atypical $ANCA^+$ patients were eventually determined to be CD, but clinically showed a UC-like CD phenotype. Remarkably, during the 9.9 yr follow-up, 48.5% of the patients did not show antibodies against ASCA or atypical P-ANCA. In 85% of these seronegative patients the diagnosis remained indeterminate. In contrast, 48% of the seropositive patients became CD or UC on follow-up. Adding anti-OmpC and anti-I2 to the serologic panel in patients with IC did not add diagnostic clarification [65].

Several groups have studied whether atypical P-ANCA and ASCA are subclinical markers of IBD in families. Some studies [66,67] showed that presence of atypical P-ANCA occurred frequently in healthy first-degree relatives of UC patients, whereas other studies were not able to confirm this observation [68,69]. ASCA positivity was obviously found at a higher rate in unaffected first-degree relatives of CD patients than in the general population (20-25% vs. 5%) [70,71]. More recently, increased anti-OmpC expression in the unaffected family members of CD patients were reported providing evidence that anti-OmpC is a familial trait that appears to be determined by genetic factors. Moreover, there were increased level of serum expression in unaffected relatives compared with healthy controls even in those individuals with expression falling within normal range, thus underscoring the fact that seroreactivity to microbial antigens is a quantitative trait [72].

Israeli et al [73] demonstrated that the presence of ASCA and atypical P-ANCA in healthy subjects can predict for IBD before the emergence of overt clinical manifestations. Serum samples were obtained systematically and stored from 5% of all military recruits. ASCA were detected in 31% of CD patients before clinical diagnosis. The mean interval between ASCA detection and diagnosis was 38 months. There was no ASCA positivity in control population. Atypical P-ANCA was present in 25% of patients with available sera before the diagnosis of UC. None of their 24 matched controls were positive.

Association with Disease Phenotypes and Progression

The occurrence of atypical P-ANCA in UC is associated with a characteristic clinical appearance and represents a distinct subgroup which is often characterized by specific HLA markers. These patients have a higher probability to develop a severe left-sided ulcerative colitis, which is more resistant to treatment than the usual case. The disease has a more aggressive course requiring surgery earlier in the course of the disease [74]. Some authors suggest that pouchitis develops more frequently after ileal pouch anastomosis [75], whereas others were not able to confirm this observation. The presence of atypical P-ANCA identifies a subgroup of CD patients characterized by “UC-like” colitis; the inflammation usually involves the left side of the colon and the response to therapy is generally good [55]. We could not confirm these findings. In our study patients with CD, who were atypical P-ANCA-positive, had no particular clinical features compared with patients who were P-ANCA-negative. Furthermore, the serological profile did not predict disease phenotype in UC [20].

The atypical P-ANCA in CD patients associated with later age of onset and a relative decreased incidence of complications such as stricture and/or perforation [76,77].

Since earlier cross-sectional study of *Vasiliauskas et al*.[16], several authors found consistently that phenotype and the disease course of CD are heavily dependent on the presence and extent of the serologic response targeted against various microbial antigens in multiple geographically distinct CD cohorts. In patients with a ASCA+ (IgG and/or IgA)/ atypical P-ANCA- phenotype, small bowel involvement (with or without colonic disease) is more typical than the pure colonic disease (68-76% vs. 34-46%)[20, 27, 78,79]. ASCA positivity predicts a more aggressive disease course with a higher rate of complications. ASCAs have been associated with stricturing and penetrating type of disease as opposed to the inflammatory one and a higher risk of small bowel resection[16, 20, 74, 80]. Several studies suggest that ASCA positivity is associated with an earlier onset of disease[16, 20, 25]. ASCA IgA positivity in children may represent a higher risk for relapses (OR 2.9 [95%CI 1.33-6.33]) [81]

The presence of anti-OmpC in adult CD patients is associated with an increased prevalence of the penetrating form only [33, 39, 80, 82] while in children both the penetrating and stenosing forms [83] are more frequent. Moreover, antibody positivity may lead to a more aggressive course of disease and a higher risk for surgical interventions.

Like ASCA and anti-OmpC, anti-I2 also appears to be associated with an increased risk for complications in adult CD patients. It is an independent risk factor for the development of the stenosing form and the need for surgical interventions [33, 39, 80, 82].

The number of antibodies produced against these microbial antigens in CD shows a positive correlation with the severity of the disease course ("serology dosage effect"). *Mow et al.*[80] analyzed 303 patients retrospectively and found that simultaneous presence of 3 antibodies (ASCA, anti-OmpC and anti-I2) results in an increased risk of complications (stenosing form [72% vs. 23%], penetrating form [58.7% vs. 27.9%]) and need for surgical intervention [72% vs. 23%]), as compared to the seronegative group. When all three antibodies are present, the OR is 8.6 (95%CI [4.0-18.9]). These findings were confirmed by a similar study in a Scottish CD cohort [82]. In addition to qualitative correlations, quantitative correlations with serologic responses to ASCA, anti-OmpC, anti-I2 are also present. Patients expressing serologic markers at high titer are more likely to have complicated small bowel CD[80].

Recently, anti-CBir1 antibody has been added to the armamentarium of available serologic markers that can be measured. Two studies[39, 40] demonstrated that the anti-CBir1 antibody is associated with ileal involvement in adult CD patients independently from other serologic markers, and it predisposes for the development of both stenosing and penetrating forms.

These findings were largely corroborated by two recent large scale studies[32, 35] examining the association between a new set of antibodies directed against anti-glycans (gASCA, ALCA, ACCA, and AMCA) and a more universal antibody against anti-OmpC. The presences of these antibodies are closely related to a complicated disease phenotype and need for surgery. The number and the magnitude of serological response were also associated with ileal, non-inflammatory disease and the risk for surgery.

Studies evaluating disease associations with seroreactivity to microbial antigens are limited by different facts. Firstly, the ideal way to address this issue would be to study patients prospectively, early in the course of their disease before the development of complications. However, the above mentioned studies were cross-sectional ones representing IBD patients who have already developed (or not) certain disease complication [84]. We now have two 2 key studies in this regard [83, 85] confirming the findings in cross-sectional studies. In a prospective pediatric cohort (n=196) *Dubinsky et al.*[83] found that the presence and magnitude of immune responses to microbial antigens (ASCA, anti-OmpC, anti-I2 and anti-CBir1) early in the course of the disease [median 9.4 months postdiagnosis] are significantly associated with more aggressive disease phenotype. The risk of developing penetrating and/or stricturing CD was increased 11-fold in those individuals with immune responses to all four microbial antigens compared to seronegative cases (95%CI [1.5-80.4]). Moreover, they demonstrated that the time to develop a disease complication during the 18 months of follow-up period is significantly faster in those children who have a serologic response against at least 1 antigen. There is a difference between the cohort studies performed in children and adults indicating differences as to which immune response has the greatest effect on the course of the disease in children and adults. The reason for this difference is not yet understood.

Amre et al.[85] also studied a cohort of pediatric CD patients prospectively and they found that among ASCA+ patients the occurrence of surgical procedures was higher, and an even stronger association was found in the ASCA+/P-ANCA- patient subgroup. Survival analysis revealed that the time to first complication (fistula or abscess) was shorter for ASCA+ patients than those who were ASCA-, and for P-ANCA- patients than P-ANCA+ patients.

Secondly, important confounding factors were not tested in many of the previous cross-sectional serology studies; however influence disease phenotype (eg. disease duration and smoking habit). And the association between serological markers and disease phenotype was not corrected for these factors. Correcting phenotype-serotype associations with disease duration when multiple factors are included in the analysis can reveal that some of them are rather time-dependent variables and are not associated with the serological profile *per se*.

Thirdly, one could query whether a complication leads to an alteration in mucosal permeability and hence seroreactivity to microbial antigens. The prevalence and titer of ASCA, anti-I2, anti-OmpC, and also the presence of multiple serologic responses were more frequent after longer disease duration in previous studies [16, 82]. In our Hungarian CD cohort [32] only ACCA and anti-OmpC response, but not other anti-glycans including ASCA, was positively associated with disease duration. Similarly, the presence of ASCA in other cohorts of CD patients were also relatively constant during the course of the disease [76,77]. These observations and the finding that ASCA antibodies may appear before the diagnosis of CD and may predict the development of IBD [73] further support the importance of prospective longitudinal studies using serological markers.

One should be also aware of the fact that really striking differences in serologic response are demonstrated only in a minority of patients. About 1/4 of the patients are positive for several antibodies and have markedly elevated antibody titers at the same time. The proportion of seronegative patients, or patients being positive for only 1 antigen with low

antibody titer, is about the same. The remaining 50% of all CD patients have an intermediate phenotype based on the serologic assessments. The aim is to find new serologic markers with which we shall be able to identify certain homogenous groups of patients in this "grey zone" regarding disease progression and response to therapy.

Serologic Markers in the Follow-Up and Treatment of IBD

In patients with UC, no correlation was found between the presence and titer of atypical P-ANCA and the activity of the disease. The titer of atypical P-ANCA remains unchanged even after a colectomy [86,87]. Similarly, the presence of ASCA seems to be independent from the disease activity[76, 77]. As a consequence, neither atypical P-ANCA, nor ASCA is suitable for monitoring of the disease.

Landers et al. found that following anti-TNF-α treatment the prevalence and titer of the various antibodies (ASCA, atypical P-ANCA, anti-I2 and anti-OmpC) remained unchanged in the majority of patients [33]. Mesalazine treatment also leaves the ASCA level unchanged in active CD patients [88]. ASCA positivity remains even after steroid treatment, but the antibody titer decreases [89].

The role of the serologic response in the prediction of therapeutical effectiveness is yet to be determined. A Belgian study involving 279 CD patients failed to find any correlation between the atypical P-ANCA or ASCA positivity and the rate of response of patients given anti-TNF-α treatment. The investigators observed a generally poorer responsiveness in the case of atypical P-ANCA$^+$/ASCA$^-$ status, but the difference was not significant [90]. Similarly, significantly lower early clinical response (55% vs. 76% OR 0.4 (95%CI 0.16-0.99), p=0.049) were reported in ANCA$^+$/ASCA$^-$ UC patients after infliximab treatment by the same group [91].

Patients exhibiting serologic responses directed against various microbial antigens (OmpC and I2) should expect a higher remission rate if the budesonide treatment was supplemented by ciprofloxacin and metronidazole, while in anti-OmpC/I2 seronegative group budesonide treatment alone proved to be more effective [92]. The study brings up the possibility that certain antibiotics are more effectively used in those CD patients who present a marked immunologic response against microbial antigens. This group of patients may be the one that can be most effectively treated by manipulating the bacterial flora.

Mutations of the Serologic Markers and Receptors Taking Part in Innate Immunity

The real importance of the antibodies produced against various microbial and autoantigens may yet to be discovered. Increasing amount of evidence suggests that the hyperresponsive adaptive immunologic response to microbial antigens found in patients with CD is a reflection of the underlying immunopathogenesis of this disorder. Whether this adaptive immunologic response is reflective of acquired characteristics or has an underlying genetically determined influence is an important question. Supporting the latter possibility is

the observation that increased ASCA and anti-OmpC expression, both qualitatively and quantitatively, in unaffected relatives of patients with CD[70,71,72]. Recent findings [93] in the C3Bir mouse model – a genetic defect in innate immunity accompanying the Cdcs1 allele results in a hyperresponsive to bacterial ligands – also suggests this link. In humans, loss-of-function mutations of the innate immune gene nucleotide-binding oligomerization domain (NOD2) could results in the same phenomenon. In most[32, 94, 95] but not all previous studies[79, 80, 82] it was revealed that patients with CD carrying a NOD2 variant had a higher qualitative and quantitative serologic response than patients carrying no variants. This association was independent of the association with ileal involvement and complicated disease behavior that have been described for NOD2 variants and antimicrobial antibodies respectively. Moreover positive correlation was found between the number of mutations and the prevalence of anti-microbial antibodies indicating genetic dosage effect. In the study of *Devlin et al.*[91] unaffected relatives carrying a NOD2 variants had greater serologic response to microbial antigens than those carrying no variants further strengthen the above mentioned hypothesis. The increased expression of antibodies directed against bacterial and yeast antigens is likely a function of increased exposure of the mucosal immune system to a range of microbial antigens owing to diminished initial clearance, perhaps due to impaired secretion of defensins. Hence, a defect in muramyl dipeptide signaling via NOD2 variants could result in impaired defense against microbial species, with the subsequent development of antibodies to microbial antigens being a secondary phenomenon due to bacterial invasion and increased exposure of the mucosal immune system to a range of microbial antigens [96].

Our group found a significant, inverse association between the DEFB1 20A variant and positivity of anti-glycan antibodies, irrespective of location or disease behavior [97], but we did not find an association between NOD1/CARD4 and any of the glycan markers or conventional ASCA antibodies in CD. In contrast, *Henckaerts et al*.[95] reported that CD patients carry at least one GG-indel allele in NOD1/CARD4 had a higher prevalence of gASCA antibodies than patients who carry wild type allele. Here also, gene-dosage effect was observed. Of note, in our study the E266K variant was investigated. Finally we failed to demonstrate an association between the DLG5 R30Q variant and any of the serological markers investigated.

Mannan-binding lectin (MBL) is also a pattern recognition receptor and an important component of innate immunity. In the study of *Seibold et al*. [98], MBL deficiency was associated with ASCA positivity not only in patients with CD but also in their relatives. Lymphocytes of the patients also showed to proliferate in response to mannan. Thus, it appears that MBL deficiency could impair normal processing of mannan-expressing microbial antigens, such as those found on the cell surface of many common microorganisms. The accumulated antigens could then stimulate the immune system, and contribute to the production of ASCA and possibly the pathogenesis of Crohn's disease [99]. It should be noted, however, that a follow-up study, testing a larger cohort of CD patients failed to confirm the significant association between variant MBL genotypes and ASCA positivity. The observed trend, however, did show that the frequency of ASCA positivity was proportional to the relative deficiency of the coding genotype [100].

No association was found between variants in TLR genes and seroreactivity to microbial antigens. This is not necessarily surprising because the association between variants in TLR genes and IBD has been less consistent than the association between CD and functional variants of NOD2 gene[32, 94].

Conclusion

ASCA and atypical P-ANCA remain the best characterized markers in IBD. Unlike ASCA assays, which are generally ELISA tests and both simple to run and well-standardized, atypical P-ANCA testing is dependent on experienced personnel for both running and interpreting the test results. Results of the various assays used for the detection of atypical P-ANCA may differ significantly from each other and must therefore be compared very carefully. Individually ASCA and atypical P-ANCA tests have moderate sensitivity and specificity. Atypical P-ANCA and ASCA cannot be used for monitoring, because the antibody titers are relatively stable and do not correlate with disease activity.

Assessing both ASCA and atypical P-ANCA reactivity allows better differentiation of CD from UC and from non-CD than using the individual tests alone. The $ASCA^+$/atypical P-$ANCA^-$ phenotype is characteristic of CD, while the $ASCA^-$/atypical P-$ANCA^+$ phenotype is seen primarily in UC. Viewed together, the two assays offer a differential diagnostic which may be particularly helpful in those cases when the diagnosis of CD or UC cannot be safely established using conventional investigation methods (medical history, radiological assessments, endoscopy and biopsy). These markers can help in the assessment of about half of the patients in the indeterminate colitis (CD or UC) category. However, the presence of ASCA and other anti-glycan antibodies in patients with gastrointestinal symptoms may also indicate celiac disease.

In Crohn's disease ASCA positivity carries a higher chance of a complicated disease behavior and the need for early surgical intervention. In UC in the presence of atypical P-ANCA one can expect a dominantly left-sided disease which is often severe and resistant to therapy, and the risk of early surgical intervention is high.

Newer markers derived from various microbial inhabitants of the gut, such as Omp, I2, and CBir1, as well as various glycan markers offer new ways to stratify patients into serologic subgroups. The cumulative presence and extent of the serologic response directed against these various makers may act as prognostic indicators of the severity and behavior of the ileal disease. Patients with CD and unaffected relatives carrying variants of the NOD2 gene have increased adaptive immune response to microbial antigen supporting an underlying genetically determined influence and the importance of altered microbial sensing in the pathogenesis of the CD. The role of the antimicrobial antibodies as subclinical markers in healthy relatives of IBD patients is yet to be established.

There is considerable overlap of the reactivity of many of the new serological markers and additional studies to more fully understand the basis for their development as well as their clinical significance are required. Addition of these, as well as yet to be discovered new markers, to the serologic IBD diagnostic algorithm will likely result in incremental increases in sensitivity. Increases in sensitivity however, can often be accompanied by reductions in

specificity and this outcome must be carefully assessed and recognized. Evolution of effective serological test panels will involve sifting through the various markers to arrive at optimal diagnostic utility balanced with practical economic realities.

We have made significant progress in understanding the clinical features associated with various serologic markers in IBD. The on-going challenge is how to best utilize these new assays to provide clinically relevant information in a cost-effective manner. Assembly of logical panels of serologic markers to identify patients who are at a predicted increased risk of more severe disease and who may benefit from and early intensive monitoring and therapy to improve long-term outcome is a primary practical goal. Further prospective clinical trials will be needed to determine the evolving role and practical clinical importance of serologic assessments in IBD.

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