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TOPIC HIGHLIGHT

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New serological biomarkers of inflammatory bowel disease

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Abstract

Serological biomarkers in inflammatory bowel disease (IBD) are a rapidly expanding list of non-invasive tests for objective assessments of disease activity, early diagnosis, prognosis evaluation and surveillance. This review summarizes both old and new biomarkers in IBD, but focuses on the development and characterization of new serological biomarkers (identified since 2007). These include five new anti-glycan antibodies, anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), anti-manobioside IgG (AMCA), and antibodies against chemically synthesized (∑) two major oligomannose epitopes, Man α-1,3 Man α-1,2 Man (∑Man3) and Man α-1,3 Man α-1,2 Man α-1,2 Man (∑Man4). These new biomarkers serve as valuable complementary tools to existing biomarkers not only in differentiating Crohn's disease (CD), ulcerative colitis (UC), normal and other non-IBD gut diseases, but also in predicting disease involvement (ileum *vs* colon), IBD risk (as subclinical biomarkers), and disease course (risk of complication and surgery). Interestingly, the prevalence of the antiglycan antibodies, including anti-Saccharomyces cerevisiae antibodies (ASCA), ALCA and AMCA, was found to be associated with single nucleotide polymorphisms (SNPs) of IBD susceptible genes such as NOD2/CARD15, NOD1/CARD4, toll-like receptors (TLR) 2 and 4, and β-defensin-1. Furthermore, a gene dosage effect was observed: anti-glycan positivity became more frequent as the number of NOD2/CARD15 SNPS increased. Other new serum/plasma IBD biomarkers reviewed include ubiquitination factor E4A (UBE4A), CXCL16 (a chemokine), resistin, and apolipoprotein A-IV. This review also discusses the most recent studies in IBD biomarker discovery by the application of new technologies such as proteomics, fourier transform near-infrared spectroscopy, and multiplex enzyme-linked immunosorbent assay (ELISA)'s (with an emphasis on cytokine/chemokine profiling). Finally, the prospects of developing more clinically useful novel diagnostic algorithms by incorporating new technologies in serological biomarker profiling and integrating multiple biomarkers with bioinformatics analysis/modeling are also discussed.

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Key words: Serological biomarkers; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Anti-chitobioside IgA; Anti-laminaribioside IgG; Anti-manobioside IgG; Anti-synthetic mannoside antibodies; Multiplex enzyme-linked immunosorbent assay; Proteomics

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INTRODUCTION

Biomarkers of inflammatory bowel disease (IBD) are measurable substances in body fluids [such as blood (serological)], stool, or other parts of the body, as tools for disease diagnosis and/or prognosis. Application of IBD biomarkers is cheaper, less laborious, less invasive, and more objective compared to the endoscopy/biopsy-based approach[1]. Current IBD biomarkers include serological, fecal and genetically predisposed gene polymorphisms[2-5], as well as imaging biomarkers in conjunction

with imaging technologies such as optical, ultrasound, magnetic resonance imaging (MRI), X-ray, computer tomography (CT), position emission tomography (PET), single photon emission computed tomography (SPECT)[6-9]. Among those, fecal[10,11] and serological biomarkers, including systemic level of specific antibodies and other serum proteins[4,12-14], have been most widely explored and/or used in clinical studies. However, none of the current commercially available biomarker tests/assays, including all of those mentioned in this highlight, can be used as stand-alone tools in clinics and, therefore, can only recommended as an adjunct to endoscopy in diagnosis, and prognosis of the disease[1,15]. Considering that endoscopy is a highly resource-intensive process (involving frequent invasive, labor-intensive and expensive colonoscopic procedures), new IBD biomarkers and more comprehensive bioinformatic algorithms with multiple biomarkers are in great need.

The focus of this highlight is on new serological IBD biomarkers. However, the remarkable rapid development of IBD biomarkers in the last decade have made it impossible to separate the "old" from the "new", since multiple biomarkers, both old and new, are being integrated in clinical studies. For example, 5 serum biomarkers, including ASCA, pANCA, anti-OmpC, anti-Cbir and anti-I2 (see below), were the most widely studied in the past decade, but are still being characterized and validated for their clinical utility. Since the status of these biomarkers has been reviewed extensively elsewhere[4,12-14], they will be listed as "old" markers and only briefly reviewed, along with many other serological biomarkers reported before 2007. Anti-glycan antibodies, a newer panel of serum biomarkers, first reported in 2006[16,17] and being validated since 2007, will be one of the major "new" biomarkers in this highlight. Serum cytokines, which are among the earliest inflammatory mediators studied, but are yet to be recognized as useful IBD biomarkers, will be reviewed and discussed. The prospects of developing new serological IBD biomarkers and integrating existing ones will also be discussed, particularly regarding the application of novel molecular approaches and proteomic technologies in biomarker screening and identification, as well as novel bioinformatic analyses of clinical utilities of multiple biomarkers.

"OLD" SEROLOGICAL IBD BIOMARKERS: A BRIEF OVERVIEW

Anti-Saccharomyces cerevisiae antibodies (ASCA) and perinuclear antineutrophil cytoplasmic antibodies (pANCA) were the first extensively characterized serological IBD markers[18,19]. ASCA is more associated with Crohn's disease (CD) while pANCA is more associated with ulcerative colitis (UC)[4,13,20,21]. Three additional serum biomarkers were introduced later, including antibodies against outer membrane porin C (anti-OmpC), Pseudomonas fluorescens bacterial sequence I2 (anti-I2), and bacterial flagellin (anti-CBir 1)[12,13,22,23]. These are five most extensively studied serological biomarkers to date. Although exact data from independent studies vary, combinations of more than one of the 5 serological markers have been shown to have the most clinical value (see reviews[4,12-14,24]). For example, ASCA and pANCA together have a specificity of approximately 90% for both CD and UC[21,25-27]. These markers have been demonstrated as not only being useful for differentiating IBD *vs* healthy control or CD *vs* UC, but also as potential indicators and/or predictors for disease activity/location, disease course/complication, need for surgery, and prognosis of therapy. For example, CD patients who are positive in multiple anti-microbial antibodies (ASCA, anti-OmpC, anti-CBir, and anti-I2) have increased risk of having more complicated disease. Patients who are positive in all four of these biomarkers have 11-fold increased risk to develop penetrating and/or stricturing disease[28-32]. CD patients positive with three markers (anti-OmpC, anti-CBir, and anti-I2) are more likely to have small bowel surgery than those who were negative (72% *vs* 23%). No similar association of serotype was found with disease phenotype of UC[32].

Elevated levels of serological biomarkers were shown to be associated with IBD-susceptible gene variants. Family members of CD patients with NOD2/CARD15 3020insC variant was reported to have increased intestinal permeability, which has been positively associated with elevated serological biomarkers[33,34]. However, reports on this relationship have been inconsistent[35-38], even though more studies presented a positive association between serological biomarkers and susceptible gene variants[32,37,38]. Future studies by independent groups with larger cohorts, well-defined clinical characteristics and patient populations (such as ethnicity) are necessary to resolve this discrepancy.

Other note-worthy aspects of these serological biomarkers include their potential value as subclinical biomarkers and their inherent geographic/ethnic heterogeneity. (1) Independent studies have shown that the prevalence of ASCA positivity is significantly higher (20%-25%) in unaffected first-degree relatives of patients with CD[18,39] compared to general healthy populations (0%-10%), indicating a familial association. A much stronger indication that ASCA may be a potential subclinical biomarkers for CD came in 2005. In a serological analysis of a large serum depository, Israeli *et al* reported that ASCA reactivity was found 38 mo before clinical diagnosis in 32% of the CD patients studied[40]; (2) The diagnostic value of serological biomarkers can vary significantly among different ethnic or geographic populations. For example, both ASCA and pANCA were found to be less sensitive in Chinese and Japanese patients[41,42]. On the other hand, positivity of pANCA was shown to be higher in Mexican-American UC patients: all Mexican-Americans with UC tested had positive pANCA compared to only 40% of Caucasians[42]. These studies suggest that physicians must factor the patients' ethnic background when serological biomarkers are applied in the clinical settings.

At least two dozen non-antibody serum biomarkers have also been reported, including, C-reactive protein,

calprotectin, and PMN-elastase, soluble selectins, adhesion molecules, and procalcitonin (PCT)[4,5,43-45]. However, it is necessary to point out that most of these markers have not been extensively characterized. Many of them are also elevated in a variety of other inflammatory or pathological conditions with a low specificity to IBD. Therefore, their actual clinical value needs to be further investigated or validated.

"NEW" SEROLOGICAL IBD BIOMARKERS

New anti-glycan antibodies: ACCA, ALCA and AMCA

New diagnostic and predicting value: Three new anti-glycan antibodies were first reported as potential novel serological biomarkers in the diagnosis of IBD by Dotan *et al* in 2006 from Glycominds Ltd in Israel[16,17]. Now, as major components of IBDXTM Panel marketed by Glycominds Ltd (http://www.ibdx.net/index.html), this new set of biomarkers contains three anti-glycan antibodies, including anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), and anti-mannobioside IgG (AMCA) (Table 1). The fourth component in the IBDXTM Panel is gASCA (ASCA IgG), virtually the same as ASCA, which is the first antiglycan IBD serological biomarker identified. Since 2007, several independent studies on these anti-glycan antibodies have been reported, and their clinical utility has been validated by independent laboratories (see below). Glycan, a generic term for all molecules bearing glycosidic bonds, includes mono-, oligo- and ploy-saccharides or carbohydrates[17]. Glycans are major building blocks of cell surface components and immunogens (erythrocytes, immune cells, and microorganisms) that lead to generation of a variety of anti-glycan antibodies, including IgG, IgA, IgE and IgM, which have been demonstrated in a number of inflammatory and autoimmune diseases[16]. Mannobioside (AMCA) is a dimer of 1,3 linked mannose, and is a component of mannan from pathogenic fungi and yeast[12]. Laminaribioside (ALCA) is the building block of laminarin, a polysaccharide of the β-1-3-glucan family and is found in the cell walls of fungi, yeast, and algae[16,46]. Chitobioside (ACCA) is a component of chitin, found in the insect cuticle and cell walls of infectious pathogens such as bacteria and yeast[16]. Both β-1-3 glucans and chitin modulates the immune system by binding to receptors on neutrophils, macrophages, and natural killer (NK) cells, thereby stimulating cell proliferation, phagocytosis and cytokine secretion[47]. The resultant antibody production specifically against chitin and glucan, and their association with CD also suggests the intrinsic modulation of the adaptive immune system. However, the individual diagnostic differences between ALCA, AMCA, and ACCA have not yet been clearly established.

Using GlycoChip glycan array technology[48,49] and enzyme-linked immunosorbent assay (ELISA), Glycominds Ltd developed the new IBD serological markers (ACCA, ALCA, and AMCA)[16,17]. The initial study of these 3 new markers, which involved a total of 194 patients with CD, 162 with UC and 142 healthy controls, showed that ACCA, ALCA, and AMCA exhibited the highest discriminative capability between CD and UC[16]. Approximately one third of CD patients are positive for each of the 3 new markers[16] (Table 1). More significantly, 44% (12/27) of ASCA-negative CD patients were positive for ALCA or ACCA. Therefore, although the prevalence of each of the individual new biomarker is relatively poor (Table 1), together they are a significant complement to ASCA. In patients that were positive with one of the 3 markers, the sensitivity and specificity for diagnosis of CD were 77.4% and 90.6%, respectively. In patients with 2 or 3 of these antibodies, the specificity increased to 99.1%. Higher levels of ALCA and AMCA were significantly associated with small intestinal disease.

Ferrante *et al*[46] reported a study that involved a larger cohort, including 1225 IBD patients (913 CD, 272 UC, and 40 IC), 200 ethnically matched healthy controls, and 113 patients with non-IBD intestinal inflammation (diverticulitis, infectious colitis, ischemic colitis and pseudomembranous colitis). In this study, IBDX™ Panel (ACCA, ALCA, AMCA, and gASCA) and anti-OmpC were analyzed. 76% of CD patients are positive for at least one of the 5 markers. All antiglycan and anti-OmpC were specific for CD (80.5%-93%). The sensitivity was calculated as: gASCA = 56.4%; ALCA = 17.7%; ACCA = 20.7%; AMCA = 28.1%; and anti-OmpC = 29.1%. Among all 913 CD patients, only 13 (1.4%) were positive for all the 5 CD-associated markers. Fifty percent of CD patients (*n* = 435) who were either sASCA negative/OmpC positive (*n* = 93) or gASCA positive/OmpC negative (*n* = 342), were positive for at least one of the antiglycan markers. Sixty-seven percent of gASCA/OmpC-positive CD patients were also positive for at least one of the other anti-glycan

Table 1 New serological IBD biomarkers

		CD	UC	Control	Ref.
1	ALCA	Approximately 19%-38%	Low (approximately 7%)	Low	16, 17 18, 50
2	ACCA	Approximately 21%-40%	Low	Low	16, 17 18, 50
3	AMCA	Approximately 28%	Low	Low	16, 17 46
4	AΣMA: Anti-ΣMan3 Anti-ΣMan4	38.60% 22.10% 28.50%	Low	Low	55
5	UBE4A	46.20%	7.10%	3.30%	56
6	CXCL16	Elevated	Elevated		57
7	Apolipoprotein A-IV	Elevated in active CD			58
8	Resistin	Elevated	Elevated		59
9	PF4, MRP8, FIBA and Hpα2				68, 69
10	Cytokines/ chemokines & their receptors				

ACCA: Anti-chitobioside IgA; ALCA: Anti-laminaribioside IgG; AMCA: Anti-mannobioside IgG; AΣMA: Anti-ΣMan3 or ΣMan antibodies; ΣMan3: Synthetic Man α-1,3 Man α-1,2 Man; ΣMan4: synthetic Man α-1,3 Man α-1,2 Man α-1,2 Man; UBE4A: Ubiquitination factor E4A; CXCL16: A transmembrane protein functioning as a chemokine and a scavenger receptor.

antibodies. Of 305 sASCA/OmpC-negative CD patients, 7% were ALCA positive, 12% ACCA positive, and 13% SMCA positive. Although addition of ALCA (gASCA/pANCA/ALCA combination) resulted in only minor improvement in differentiating CD from UC compared to the classic gASCA/pANCA combination, it significantly enhanced the accuracy of differentiating IBD from healthy controls and non-IBD intestinal inflammation. Increasing levels of all 5 markers (gASCA, ALCA, ACCA, AMCA, and OmpC) were significantly associated with more complicated disease behavior, including stricture, fistula and need for surgery. However, a recent report by Simondi *et al*[50] found that, while the level of ASCA appeared to be associated with ileal disease and penetrating/structuring disease, level of ALCA has a similar trend, but did not reach statistic significance (P = 0.07 and P = 0.09, respectively). This discrepancy may arise from the smaller cohort in Simondi D's study, which involved only 265 subjects (116 CD, 53 UC, 51 healthy controls, and 45 other intestinal diseases).

Similar results on antiglycan antibodies were reported by Papp *et al*[22] in another study that involved 557 CD patients, 95 UC and 100 healthy controls. 66.2% of CD patients were positive for at least one of the 5 biomarkers tested, including gASCA, ALCA, ACCA, AMCA, and anti-OmpC, all of which were highly specific for CD (79%-100% sensitivity). The sensitivities for each of the 5 markers are gASCA = 50.4%; ALCA = 15.2%; ACCA = 11.3%; AMCA = 11.5%; and anti-OmpC = 31.2% (the 3 new markers, ALCA, ACCA and AMCA were all lower than the results of most other studies[16,17,46,50]. Overall, increasing levels of these markers were again associated with more complicated disease behavior and incidence of surgery. Among CD patients, gASCA and ALCA were associated with early disease onset (occurring at younger age (P < 0.0001 and P = 0.0012, respectively, while gASCA was associated with perianal disease (P < 0.0001) and azathioprine use (P = 0.016). However, no association was found between these serological biomarkers and gender, familial disease, smoking habit and extraintestinal manifestations (EIM). It is interesting to mention that in Simondi D's report[50], (1) among CD patients, AMCA was found to be significantly higher in women and in smokers than in men (P = 0.02) and non-smokers (P = 0.03); and (2), CD patients with at least one affected first-degree relative exhibited significantly higher levels of ALCA than those without familial cases (59.8% *vs* 34.7%, P = 0.0005), suggestive of a familial association of ALCA.

Association of the antiglycan antibodies with variants of IBD susceptible genes: NOD2/CARD15. Like ASCA[32,37,38], the newly identified antiglycan antibodies were also found to be associated with single nucleotide polymorphisms (SNPs) of IBD susceptible genes. The first study, reported by Henckaerts *et al* in 2007[51], examined the influence of mutations in several innate immune receptor genes on the development of anti-glycan and anti-OmpC antibodies in IBD, including NOD2/CARD15, NOD1/CARD4, TUCAN/CARDINAL/CARD8, Toll-like receptor (TLR) 4, TLR2, TLR1 and TLR6. The study involved 1163 unrelated IBD patients (874 CD, 259 UC, and 30 IC) and 312 healthy controls. CD patients with at least one NOD2/CARD15 variant (1) were more frequently gASCA or ALCA positive than those with no mutation (gASCA: 66.1% *vs* 51.5%, P < 0.0001; ALCA: 43.3% *vs* 34.9%, P = 0.018); and (2) had higher gASCA titers (85.7 *vs* 51.8 ELISA units, P < 0.0001). This association was independent of ileal involvement. More interestingly, a gene dosage effect was observed when positivities of antiglycan antibodies in CD patients carrying 0, 1 and 2 NOD2/CARD15 variants were compared. Anti-glycan positivity became more frequent as the number of NOD2/CARD15 mutations increased (Table 2).

Table 2 A gene dosage effect: % positivity of antiglycan antibodies in CD patients carrying zero, one and two NOD2/CARD15 variants

	CD patients with 0 NOD2/CARD15 variant	CD patients with 1 NOD2/CARD15 variant	CD patients with 2 NOD2/CARD15 variants	P	Ref.
gASCA	51.50	64.20	72.30	< 0.0001	51
	41.60	64.60	67.50	< 0.0001	22
ALCA	34.90	42.10	46.70	< 0.04	51
AMCA	9.80	14.10	30	< 0.001	22
Any antiglycan	52.40	69.70	80	< 0.0001	22

A remarkably similar gene dosage effect on gASCA was observed by Papp *et al*[22] in 2008 (Table 2) as compared to Henckaerts L's study[51]. They analyzed the association of antiglycan with NOD2/CARD15 variants from studying 557 CD patients, 95 UC and 100 healthy controls. When comparing CD patients with NOD2/CARD15 variants to those with wild type alleles, positivity of gASCA is 65.2% *vs* 41.8% (P < 0.0001), AMCA is 18.8% *vs* 9.7% (P = 0.009), and any antiglycan is 72.5% *vs* 52.5% (P < 0.0001). In addition, Papp M's report also showed the gene dosage effect on AMCA and any antiglycan antibodies (Table 2).

NOD1/CARD4, TLR2, and TLR4: In Henckaerts L's report[51], CD patients carrying one GG-indel allele in NOD1/CARD4 had a higher prevalence of gASCA than those with wild type allele (63.8% *vs* 55.2%, P = 0.014). Gene dosage effect of NOD1/CARD4 was also evident as the number of mutant alleles increased, but did not reach statistical significance. Interestingly, CD patients with at least one mutation TLR4 (D299G) had a lower prevalence of ACCA compared with TLR4 wild type (D299A) (23.4% *vs* 35%, P = 0.013), and a lower ACCA titer (39 *vs* 49 EU, P = 0.05). An inverse gene dosage effect of TLR4 was observed: the prevalence of ACCA is 34.9%, 21.1% and 9.1% in CD patients with 0, 1 and 2 NOD1/CARD4 variants, respectively. A similar inverse gene dosage effect was also seen for anti-OmpC prevalence gene in relation to the number of TLR2 mutations. Of note, other reports did not find any

significant association of TLR4 (25) or NOD1/CARD4 (variant E266K)[52] with the positivity of any antiglycan antibodies.

DEFB1: Lakatos *et al*[52] recently reported an association of the antiglycan antibodies with specific variants of β-defensin-1 (DEFB1). In this study of a sex-matched cohort of 276 CD patients and 100 healthy controls, four IBD susceptible genes were tested, including NOD2/CARD15, NOD1/CARD4, DLG5, and DEFB1. Two DEDB1 variants, G20A and C44G, were found to be inversely associated with the positivity of antiglycan antibodies. For example, 29.6% of DEFEB1 20A carriers were antiglycan positive compared to 46% positivity in non-carriers ($P < 0.038$). However, no association of antiglycan positivity was found with either DEFEB1 G52A variant or DLG5 (R30Q), although both were shown to be associated with increased risk for CD[53,54]. Furthermore, in contrast to other reports[32,51], no gene dosage effect was observed on any of the antiglycan antibodies.

Anti-synthetic mannoside antibodies: Evidence for the existence of a new subset of antiglycan antibodies

Based on the chemical structure of mannose epitopes for ASCA, the antibodies against mannose residues [Man α-1,3 (Man α-1,2 Man) 1-2] that are the most widely recognized biomarkers for CD, Vandewalle-El Khoury *et al* recently[55] chemically synthesized (∑) two major oligomannose epitopes, Man α-1,3 Man α-1,2 Man (∑ Man3) and Man α-1,3 Man α-1,2 Man α-1,2 Man (∑ Man4). Their goals were to test the immuno-reactivity of the ∑Man3 and ∑Man4 with specific serum antibodies (termed "A∑MA" for anti-synthetic mannoside antibodies) and compared A∑MA with ASCA for their values as serological biomarkers for CD. An impressively large cohort was used in the study, including a total of 1365 subjects (772 CD, 261 UC, 43 IC and 289 controls).

Overall, while the specificity of A∑MA for CD was quite similar to that of ASCA (89% *vs* 93%), the specificity was lower (38% *vs* 55%). 16% of CD patients were positive for both ASCA and ∑Man3, and 24% positive for ∑Man4. Interestingly, 11% of ASCA-negative CD patients were A∑MA positive (5% for ∑Man3, 4% for ∑Man4, and 2% for both). Together 24% of CD patients who were negative for ASCA and/or other CD-associated serological biomarkers were positive for A∑ MA, suggesting a previously unrecognized new subset of anti-mannose antibodies are present in patients with CD. Therefore, it is conceivable that a combination of A∑MA with ASCA, ACCA, ALCA and AMCA would significantly increase the diagnostic value of the existing panel of antiglycan biomarkers.

A∑MA was analyzed for its predictive value for the evolution of IC patients as well as the involvement of disease location of CD (small bowl *vs* colon). Twenty IC patients (out of a total of 43) evolved to a final diagnosis of CD ($n = 11$; UC = 7; UC-like CD = 2). Among 11 patients with final diagnosis as CD, one was ASCA-positive/A∑MA-negative, two were ASCA-positive/A∑MA-positive, and three were ASCA-negative/A∑MA-positive. None of the UC patients were A∑MA-positive. Therefore, A∑MA was more sensitive (45% *vs* 27%) and more specific (100% *vs* 71%) than ASCA for predicting evolution of IC toward CD. As for the predictive value of disease location, although positivity of A∑MA had no association with any particular disease phenotype, among the ASCA-negative CD patients, A∑MA positivity was significantly associated with colonic involvement. This indicates that A∑MA may provide new diagnostic value to colonic CD, for which ASCA is less frequently detected.

Other serum/plasma biomarkers

Ubiquitination factor E4A (UBE4A): By screening a phage library from normal terminal ileum with sera from patients with CD, Sakiyama *et al*[56] identified a strongly immunoreactive cDNA clone encoding the C-terminal subunit of the UBE4A, a U-box-type ubiquitin-protein ligase. To investigate the specificity of the serum anti-UBE4A autoantibodies in CD patients *vs* UC patients *vs* healthy controls, a GST-C-terminal UBE4A fusion protein was made, and used to test the immunoreactivity of sera from 39 patients with CD, 28 with UC, and 60 healthy controls. The prevalence of anti-UBE4A IgG was significantly higher in CD than that in UC or healthy controls (46.2% *vs* 7.1% *vs* 3.3%, respectively; $P < 0.0006$) (Table 1). The levels of anti-UBE4A IgG were correlated well with the disease activity ($P < 0.0001$). More interestingly, higher level of anti-UBE4A IgG was associated with complicated disease behavior (stricturing and penetrating) ($P = 0.0028$), and patients positive with anti-UBE4A IgG were more likely to undergo surgery ($P = 0.0013$). Although UBE4A expression was low in the cytoplasm of enterocytes and goblet cells, immunohistological analysis showed that UBE4A expression was highly elevated only in enteroendocrine cells of ileal, mucosa from CD patients, but not in normal subjects. It was speculated that production of anti-UBE4A autoantibodies might be a result of increased expression of UBE4A in the inflamed ileal mucosa. The exact role of UBE4A elevation and production of anti-UBE4A autoantibodies in the pathogenesis of CD remains to be determined.

CXCL16, apolipoprotein A-IV, and resistin: These newly reported serum/plasma IBD biomarkers were reported by the same working group in Germany[57-59]. It appears that the same cohort of study subjects was used for all three studies, or at least cohorts might have some overlapping subjects. Further validation study of these markers by other independent laboratories is necessary to evaluate their usefulness.

CXCL16 (Table 1), an intriguing transmembrane protein composed of an extracellular chemokine domain fused with a mucin stalk that extends through cell surface, functions as a chemokine and a scavenger receptor and has been implicated in various inflammatory diseases. It becomes soluble after being cleaved by metalloproteinase ADAM 10 and exerts chemokine functions[60].

Lehrke *et al*[57] reported that CXCL16 could potentially be a surrogate IBD biomarker after having examined the serum levels of soluble CXCL16 in a cohort of 239 patients with CD, 114 UC, and 144 healthy controls. Soluble CXCL16 levels were found to be the highest in CD patients ($P < 0.001$) compared with UC and healthy controls. UC patients had a relatively modest, but significant elevation of CXCL16 compared with healthy controls ($P < 0.001$). No significant difference was seen between active and inactive state of CD or UC.

Apolipoprotein A-IV (Table 1), a structural component of intestine derived triacylglycerol-rich chylomicron particles with anti-oxidant, anti-atherogenic, and anti-inflammatory properties, has been recently shown to inhibit DSS-induced mouse colitis[61]. For this reason, Broedl *et al*[58] tested if plasma level of apolipoprotein A-IV was associated with IBD, and found that it was inversely associated with disease activity and CRP levels in patients with CD (but not UC) ($P < 0.005$). However, since the actual difference in the level of apolipoprotein A-IV between active and inactive CD is quite small, the clinical value of this marker remains to be determined.

Resistin (Table 1), also known as adipocyte secreted factor or FIZZ-3, is a peptide hormone that is associated with multiple inflammatory conditions[62]. Studies by Konrad *et al*[59] showed that patients with both CD and UC had significantly higher plasma levels of resistin compared to healthy controls ($P < 0.0001$). The levels of resistin in both CD and UC were significantly associated with white blood cell count ($P < 0.0001$), CRP ($P < 0.0001$), and disease activity ($P < 0.0001$).

Application of proteomic and infrared spectroscopic technologies in serological IBD biomarker profiling

The rapid development of proteomic technologies recently has revolutionized the way and capacity by which biomarker discovery is performed[63-67]. Current proteomic methodologies include three sub-categories: mass spectrometry (MS)-based technologies, array-based technologies and imaging MS (see review[66]). Blood is the most explored source for disease biomarkers by proteomics[63-65,67]. Proteomics of IBD was recently reviewed[80].

SELDI-TOF-MS and MALDL-TOF-MS: Unlike the quests for biomarkers of other major diseases (such as cancers), application of proteomic technologies in IBD biomarker discovery is only in its infancy. So far (as of June 17, 2008) only three full original MS-based reports are available, of which, two used SELDI-TOF-MS by Merville/Louis groups[68,69] and one used MALDI-TOF-MS by Roda's group[70]. Using SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometer), Meuwis *et al*[68] analyzed protein profiles of 120 serum samples collected from a cohort of 30 CD, 30 UC, 30 inflammatory controls and 30 healthy controls. Multivariate analysis generated models that could classify samples with minimum 80% sensitivity and specificity in discriminating groups of patients. Four peptides were identified from potential peaks that could best discriminate the four groups, leading to the identification of 4 serum biomarkers, including platelet aggregation factor 4 (PF4), MRP8 (S100A8), FIBA (a peptide released during clotting from fibrinogen precursor) and Hpα2 (haptoglobin α2) (Table 1). The diagnostic value of these markers remains to be further examined. Using similar approaches, the same group piloted a study of sera from 20 CD patients who showed either response or no response to infliximab[69]. PF4 was again identified as a potential marker. The intensity level of SELDI peak in which PF4 was identified was inversely associated with infliximab non-responders. Unfortunately, such association could not be confirmed by ELISA measurement of PF4. Also, PF4 did not exhibit a significant correlation with other disease markers (sCD40L, IL-6, and CRP) or CDAI, casting doubt that PF4 probably would have any clinical diagnostic value.

The third MS-based profiling of serum IBD markers was reported by Nanni *et al*[70] using MALTI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight-Mass Spectrometer). The study, which involved a small cohort (15 CD, 26 UC and 22 healthy controls), found the revered-phase extraction and selection of 20 m/z value gave the best overall predictive value (96.9%). In another study, reported at Digestive Disease Week (DDW) 2008, Subramanian *et al*[71] analyzed sera from a cohort of 62 UC and 48 CD by SELDI-TOF MS. Biostatistical analysis identified 12 discriminative peaks, with specificity and sensitivity approximately 95% (compared to 80.9% of the sensitivity of ASCA for CD and 64.5% of pANCA for UC). Four serum proteins were identified as inter alpha trypsin inhibitor 4, apolipoprotein C1, platelet activated factor 4 variant, which are expected to be further analyzed for their clinical utility.

Protein and antibody arrays/chips: Compared with other proteomic approaches, protein/antibody array (or chip) array technologies offer the advantages of being highly specific with high-throughput nature and capacity. The application of these technologies in IBD biomarkers discovery has just begun, and thus, the data are limited. Since 2006, our laboratory began using proteins that were robotically spotted on array slides as bait to screen serum IBD biomarkers[72]. Since the currently known major serological IBD biomarkers or antibodies are against microbes or protein of human origin, including ASCA, ANCA, OmpC, Cbir, and the new antiglycan antibodies, we hypothesized that disease-specific antibodies to microbial or to human protein (autoantibodies) are present in patients' sera (manuscript in preparation). We found to our surprise that human sera contain antibodies immuno-reactive to hundreds of proteins from *E. coli*, yeast, and even humans. The numbers and immunoreactivity of these antibodies vary greatly among IBD patients and even among healthy individuals. From our experiences, when protein arrays are used, the serum quality must be high, the screening process must be standardized, and sufficient number of subjects (at least approximately 30 per comparing group) should be included. This results in a high cost of experiments due to the expense of the commercial protein- or antibody arrays (unless made using assembled robotic ar-

rayers). One presentation by Vermeulen *at al*[73] at DDW 2008 reported a study of using commercial human protein arrays to profile serum IBD biomarkers from a very small cohort of subjects (10 UC, 15 CD and 5 healthy controls). They found that 75 proteins reacted more strongly with sera from IBD than those from healthy controls, while reactivity of another 88 proteins was just opposite. One identified antigen, described as an autoantigen in IBD was pleckstrin homology-like domain, family A, member 1 (Phla1). Validation experiment using a larger cohort of subjects found that approximately 46% of IBD [UC: 42.8% (27 of 63); CD: 50.0% (33 of 66)] were positive for anti-Phla1 antibodies, compared to 28.7% healthy controls (19 of 66) and 33.3% non-IBD gastrointestinal controls (22 of 66). Therefore, the discriminative power of this anti-Phla1 for CD *vs* UC or IBD *vs* controls is poor.

So far, antibody arrays were used in only one report by Kader *et al*[74] for identifying IBD serum biomarkers. In this study, antibody arrays containing 78 cytokines, growth factors, and soluble receptors were used to screen 65 patients with CD and 23 with UC. Univariate analysis found that the levels 4 cytokines (PLGF, IL-7, IL-12p40, and TGF-β1) were significantly elevated in patients with clinical remission compared to active disease ($P < 0.01$). However, only the difference in IL12p40 reached statistical significance ($P < 0.02$).

Fourier transform near-infrared spectroscopy: Haas *et al*[75] reported at DDW 2008 a new application of Fourier Transform Near-Infrared Spectroscopy (FT-NIR) in serum biomarker profiling. Specific spectra or fingerprints of serum samples from 139 patients with CD and 120 with UC were obtained by FT-NIR, and analyzed by Artificial Neural Networks (ANN) and cluster analyses. ANN-analysis showed that the sensitivity, specificity and accuracy for IBD *vs* healthy controls were 94.5%, 99.1%, and 96.7%, respectively, compared to cluster analysis (71.8%, 66.6%, and 70.1%). Cluster analysis correctly identified 80% of UC and 61.5% of CD, while ANN-analysis was 69.8% and 91.8%, respectively. This proof-of-concept study suggests a potential usefulness of this technology in identifying serum "fingerprints" as serological biomarkers for IBD diagnostics.

Serum cytokines/chemokines and their receptors

Serum cytokines and their soluble forms of receptors have been extensively studied, both as markers for IBD diagnosis and as molecules for IBD pathogenesis, as well as IBD therapeutic targets[4,76-78]. A long list of cytokines/chemokines and their receptors have been shown to up-regulated in active and even inactive IBD, including IL-1α, IL-1β, IL-1R (R: receptor), IL-1Ra (Ra: receptor antagonist), IL-2, sIL-2R (s: soluble), IL-6, IL-6R, IL-7, IL-8, IL-10, IL-12, IL-15, IL-16, IL-17, IL-18R, IL-27, IFN-α, IFN-β, TGF-β, TNF-α, TNF-αR, as well as most chemokines. However, the diagnostic value of cytokines/chemokines has been limited, at least in part due to studies based on the analysis of individual or only a few selected cytokine/chemokines. The ideal situation would be to profile a large number of serum cytokines/chemokines from well characterized cohorts. An example of this is the utility of a more robust and high-throughput multiplex sandwich ELISA (which allows simultaneous analysis of up to 100 analytes), in conjunction with biostatistical analysis tools [such as that of discriminant functional analysis (DFA) and multidimensional scaling (MDS)], as we and others have described[79-83]. As a proof-of-concept in the context of IBD, we recently analyzed 16 serum cytokines by multiplex ELISA from a cohort of 64 mice with or without induced experimental colitis[84]. Distinctive disease-specific cytokine profiles were identified with significant correlations to disease activity and duration of disease. Our data showed that CD-like TNBS colitis exhibits heightened Th1-Th17 response (increased IL-12 and IL-17) as the disease becomes chronic. In contrast, UC-like DSS colitis switches from a Th1-Th17-mediated acute inflammation (increased TNFα, IL6, IL-17 and KC) to a predominant Th2-biased chronic inflammatory response (increase in IL-4 and IL-10 with concomitant decrease in TNFα, IL6, IL-17 and KC). DFA identified 5 discriminatory cytokine biomarkers (IL-6, 12, 4, 17, INF-γ) that can sufficiently distinguish healthy controls from diseases, and one disease type from another[84]. A pilot study profile of 17 human cytokines/chemokines from a small cohort of 33 IBD patients (19 CD and 14 UC) with 33 matched healthy controls using multiplex ELISA also identified patterns of cytokines/chemokines that were correlated with disease phenotypes (CD *vs* UC) and severity[82]. A similar human cytokine profiling study using 24 cytokines/chemokines from a larger cohort of 400 IBD patients (with various levels of disease severity) is near completion. It will be interesting to see if the cytokine profiles can: (1) enable disease subtype stratification; (2) correlate with disease severity; and (3) if the profiles that were identified in murine experimental colitis would be similar to those of human IBD.

CONCLUSION

It is evident that while current serological IBD biomarkers are useful, their clinical utility has been limited. New technologies, such as those described in this highlight, demonstrate the significant potential for identifying previously unrecognized IBD biomarkers. Future direction is predicted to be, in addition to the continuation of ongoing efforts in developing novel biomarkers using conventional and new technologies, the integration of multiple biomarkers with extensive bioinformatics analysis/modeling. This will be the key to eventually developing specific "endpoint-oriented" serological biomarker kits. These may include, but not be limited to, individual biomarker sets that are specific for one or more of the following: (1) differentiating CD *vs* UC *vs* normal *vs* other non-IBD gut diseases that share some similar clinical presentations (such as abdominal pain and diarrhea in infectious colitis or IBS); (2) predicting IBD risk (before disease onset; subclinical biomarkers) and disease course (risk of complication and surgery); (3) predicting therapeutic efficacy even before initiation of specific

medication(s); and (4) monitoring therapeutic efficacy and predicting relapse. One can envision that such kits will rely on "integrated algorithms", rather than absolute differences, to enhance the accuracy of diagnosis and/or prognosis of IBD.

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