

## PAPER

# Anti-glycan antibodies as biomarkers for diagnosis and prognosis

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Glycans (sugars or carbohydrates) are predominant surface components of cells such as erythrocytes, immune cells and microorganisms. As such, they give rise to high levels of anti-glycan antibodies of all classes. Antibodies to certain defined mono, di and oligosaccharides that are common in bacterial, fungal and parasite cells exist in human sera and can be profiled using glycan arrays. The use of glycan arrays for systematic screening of blood samples from multiple sclerosis (MS) and Crohn's disease (CD) patients in versus to blood samples from control groups, have lead to the discovery of a few anti glycan antibodies biomarkers enabling diagnosis and prognosis in MS and CD patients. Anti-Glc( $\alpha$ 1,4)Glc( $\alpha$ ) IgM antibodies were found to be specific for MS patients, enabling differentiation between MS patients and patients with other neurological diseases, with 54% sensitivity and 85% specificity. Anti-Glc( $\alpha$ 1,4)Glc( $\alpha$ ) IgM were found to be predictive for the conversion of patients in first acute neurological event to clinically defined MS. Anti-laminaribioside (ALCA), anti-mannobioside (AMCA) and anti-chitobioside (ACCA) antibodies were found to be specific for CD. The combined use of these antibodies enables improved diagnosis of CD versus ulcerative colitis and other gastrointestinal diseases, as well as stratification of CD patients with a more complicated disease and high risk for surgery. Anti-glycan antibodies profiling (AGAP) is a new and promising approach for development of biomarkers for diagnosis and prognosis. *Lupus* (2006) 15, 1–10.

**Key words:** antibodies; Crohn's disease, diagnosis; glycan-array; multiple sclerosis; prognosis

## Introduction

There is a pressing need to develop new biomarkers that will serve at different stages of drug development and as diagnostics tools. Currently, the spot-light in research is mostly centered on genetic markers. However, while the detection of changes in levels of mRNA provides useful information, only a moderate correlation exists between the cellular content of specific mRNAs and their corresponding polypeptides. Furthermore, expression analysis lacks an advantage in post-translational modification of proteins, such as phosphorylation, acetylation and glycosylation, which modulate protein function and thus, the regulation of cell function. The development of post-genomic technologies has consequently recently gained momentum.<sup>1</sup> Antibody arrays are used to screen samples for well characterized proteins,<sup>2</sup> and protein-chips<sup>3–5</sup> and gel separation technologies<sup>6</sup> are used in large scales in

combination with mass-spectrometry to analyse total protein content. In parallel, techniques for studying specific post-translational modifications are being developed.<sup>7,8</sup> The field of glycobiology became especially active in the last few years yielding a number of publications describing techniques for the measurement of glyco-proteins,<sup>9,10</sup> and sugar arrays in analysing glycan interactions with proteins and cells.<sup>5,11</sup> Glycans, due to the large number of saccharide building blocks and the variety of linkages between them, have an enormous potential to carry information – far exceeding those of nucleic acids or proteins.<sup>12,13</sup> These glycans are displayed on macromolecules and the surface of cells where the information they encode is deciphered by glycan binding proteins in numerous processes such as the antigen recognition machinery, bacterial and viral adhesion to host cells and evasion from host immune system, and protein folding, stability and trafficking.<sup>14–18</sup> Certainly, both cellular and humoral immune responses rely heavily on interactions between glycans and glycan binding proteins, be it lectins involved in cell–cell interactions,<sup>19</sup> lectins of the innate immune system,<sup>15</sup>

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or antibodies recognizing sugar-antigens on surfaces of pathogens. These antibodies have been shown to constitute a substantial fraction of total serum IgG and IgM in healthy individuals,<sup>20</sup> but have also been implicated in a number of autoimmune diseases, most notably in neuropathies like Guillain-Barre syndrome.<sup>21</sup> Naturally occurring serum anti-glycan antibodies are therefore may be good candidates for biomarkers in inflammatory and autoimmune disease.

'Glycan' is a generic term describing molecules with glycosidic bonds, including sugar (mono-saccharides, oligosaccharides, polysaccharides or carbohydrates). In contrast to oligo-nucleotide and protein whose building blocks, are connected in a linear fashion, glycans tend to be branched structures. Glycans are predominant surface components of cells such as erythrocytes, immune cells and microorganisms and as such give rise to high levels of anti-glycan antibodies of all classes (IgG, IgM, IgA and IgE).

The use of a glycan library displayed on solid phase as an array, offers the ability to characterize the interaction between glycan binding proteins, lectins or antibodies, and a diverse library of glycans in a fast and robust way. Recently several approaches for construction of glycan arrays for analysis of glycan binding proteins specificity<sup>22–26</sup> emerged. Each format differs in the type of glycans and the manner in which they are displayed. Some use non-covalent attachments to plastic or nitrocellulose membrane,<sup>26–32</sup> and others use covalent attachments to plastic, gold, or glass.<sup>11,22,23,26,33–36</sup> The glycans are displayed in a large range, from a limited amount of five to 45 exemplary structures representing terminal sequences on glycoprotein or glycolipids glycans,<sup>26,27,31–36</sup> to libraries of up to 200 defined glycans, proteoglycan fragments, and microbial polysaccharides.<sup>28–30</sup> The major obstacle in developing diverse libraries of glycans is that in contrast to oligo-nucleotides and oligopeptides, for each specific glycan structure, a unique synthesis pathway (chemical or enzymatic) have yet to be developed. The GlycoChip<sup>®</sup> glycan array technology we developed was successfully applied to profiling the glycan binding specificities of lectins,<sup>22</sup> whole intact cells displaying lectins on their surface,<sup>11</sup> as well as polyclonal human anti-glycan antibodies.<sup>22</sup>

Following, we will review the work done in the recent years regarding: anti-glycan antibodies profiling (AGAP) in healthy populations and in patients with autoimmune inflammatory diseases. The use of glycan arrays for systematic screening of blood samples from multiple sclerosis (MS) and Crohn's disease (CD) patients opposed to blood samples from control groups, have lead to the discovery of anti-glycan antibodies biomarkers enabling diagnosis and prognosis in MS<sup>37,38</sup> and CD<sup>39–41</sup> patients.

## Anti-glycan antibodies in normal populations

Each individual human, as part of its adaptive immune arm, have circulating antibodies towards vast repertoire of non-self glycan structures existing on bacterial, fungal and parasite cells. Amongst the first well-studied anti-glycan antibodies in humans are the anti-blood groups: antibodies. Individuals of a specific blood group type have high levels of anti-glycan antibodies to the reciprocal type of blood group glycans, anti-GalNAc( $\alpha$ 1,3)[Fuc( $\alpha$ 1,2)]Gal( $\beta$ ) for blood type A or anti-Gal( $\alpha$ 1,3)[Fuc( $\alpha$ 1,2)]Gal( $\beta$ ) for blood type B.<sup>42</sup> Another well characterized anti-glycan antibody in healthy population is anti-Gal( $\alpha$ 1,3)Gal antibodies, that exists in humans, apes and old world monkeys, and constitutes approximately 1% of circulating IgG in human serum.<sup>43</sup>

### *Anti-glycan antibody profile (AGAP) in human IgG pool*

Applying the analysis of the anti-glycan antibodies binding profile of human IgG pool (IgG affinity-purified from sera collected from 10 000 healthy individuals) to 34 mono- and oligosaccharides array (see Table 1 for glycans included in the array), has shown that the strongest signals were recorded for IgG antibodies against  $\alpha$ -GlcNAc,  $\alpha$ -L-Rha, medium levels for  $\beta$ -GlcNAc and Gal( $\alpha$ 1,3)Gal( $\beta$ 1,4)GlcNAc whereas lower levels were observed against  $\beta$ 4-linked oligosaccharides of glucose,  $\alpha$ -Gal and GlcNAc( $\beta$ 1,4)GlcNAc( $\beta$ )<sup>22</sup> (see Figure 1).

### *Anti-glycan antibody profile (AGAP) in a normal human population*

We profiled the levels of anti-glycan antibodies of IgG, IgA, and IgM subtypes, against seven mono- and oligosaccharides in 200 sera samples taken from apparently healthy blood donors. The AGAP was done as previously described<sup>22</sup> with a modification: instead of using biotinylated protein A for detection of bound Ig we used biotinylated goat anti-human Ig antibody, of IgG, IgA and IgM subtypes, at 2.8  $\mu$ g/mL, 3  $\mu$ g/mL and 0.9  $\mu$ g/mL, respectively, for IgG, IgA and IgM determination. The distribution of anti-glycan antibodies levels is displayed in Figure 2. The AGAP magnitude of subclasses IgG and IgA were higher than that of IgM. The female sub-population had statistically significant higher levels of anti-glycan antibodies than the male sub-population, but the AGAP of the two sub-populations was identical (not shown). The anti-glycan antibodies of the population tended to fit a lognormal distribution. It is evident that considerable variation in anti-glycan antibody levels exists between individuals

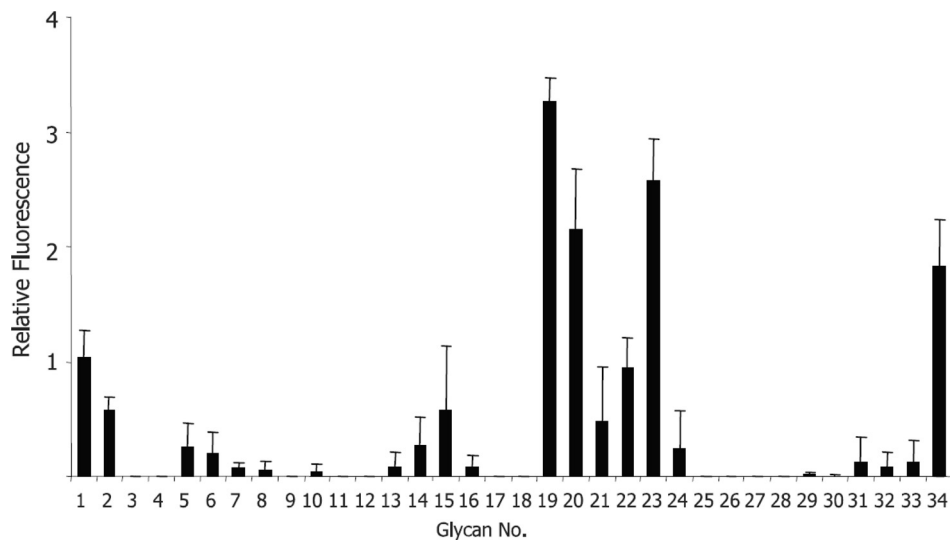
**Table 1** Glycan displayed on glycan array

Glycan no.	Glycan structure
1	$\alpha$ -Gal
2	$\beta$ -Gal
3	Gal( $\beta$ 1,3)GalNAc( $\alpha$ )
4	Gal( $\beta$ 1,3)GlcNAc( $\beta$ )
5	Gal( $\beta$ 1,4)Glc( $\beta$ )
6	Gal( $\beta$ 1,6)Gal( $\beta$ )
7	$\alpha$ -GlcNAc
8	$\beta$ -GlcNAc
9	$\alpha$ -Fuc
10	$\beta$ -Fuc
11	$\alpha$ -Glc
12	Glc( $\alpha$ 1,4)Glc( $\alpha$ )
13	Glc( $\alpha$ 1,4)Glc( $\beta$ )
14	$\beta$ -Glc
15	Glc( $\beta$ 1,4)Glc( $\beta$ )
16	Glc( $\beta$ 1,4)Glc( $\beta$ 1,4)Glc( $\beta$ )
17	Glc( $\beta$ 1,4)Glc( $\beta$ 1,4)Glc( $\beta$ 1,4)Glc( $\beta$ 1,4)Glc( $\beta$ )
18	Glycerol (background)
19	$\alpha$ -GlcNAc
20	$\beta$ -GlcNAc
21	GlcNAc( $\beta$ 1,3)GalNAc( $\alpha$ )
22	GlcNAc( $\beta$ 1,4)GlcNAc( $\beta$ )
23	$\alpha$ -L-Rha
24	$\beta$ -GalA
25	$\alpha$ -Man
26	$\beta$ -Man
27	$\alpha$ -Neu5Ac
28	$\alpha$ -L-Araf
29	$\beta$ -GlcA
30	$\alpha$ -Xyl
31	$\beta$ -Xyl
32	Gal( $\beta$ 1,3)[GlcNAc( $\beta$ 1-6)]GalNAc( $\alpha$ )
33	Gal( $\beta$ 1,4)GlcNAc( $\alpha$ )
34	Gal( $\alpha$ 1,3)Gal( $\beta$ 1,4)GalNAc( $\beta$ )

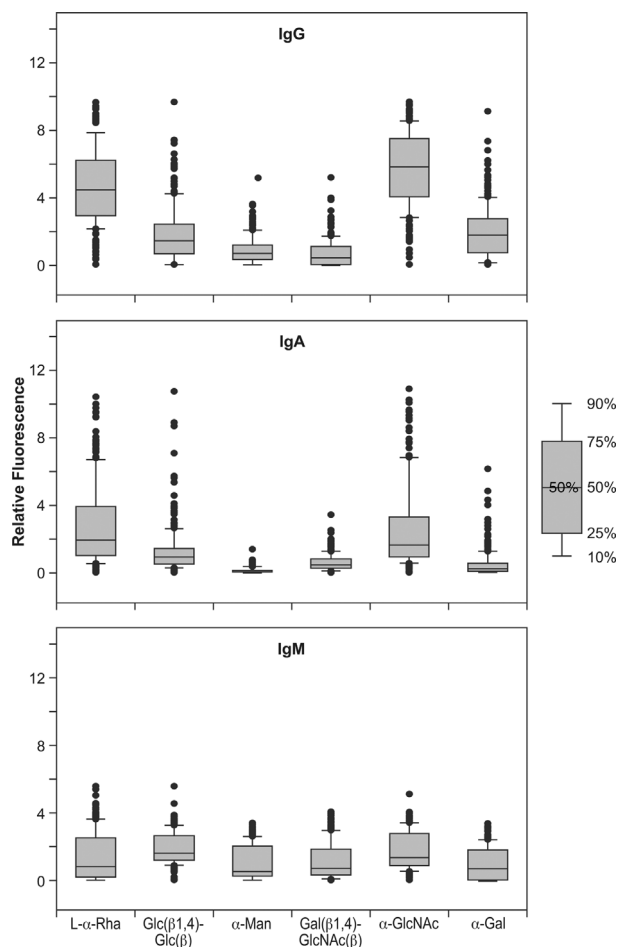
within the population examined, a fact that suggests the existence of individual AGAPs, but, limits the search of markers to anti-glycan antibodies present in low amounts.

#### Temporal range of anti-glycan antibody levels

When considering any biological parameter for the use as a surrogate biomarker, it is obviously a prerequisite that its level is temporally stable. Thus, we followed the serum levels of IgG, IgA and IgM anti- $\alpha$ -Rha, anti- $\alpha$ -GlcNAc and anti-celotriose antibodies, as described above, in seven healthy volunteers during the course of 13 weeks (Figure 3). In general, the serum antibody concentrations vary between the different individuals, but are quite stable over time. For example, sera #1961 and #1962 have extremely high and temporally stable relative levels of IgA anti- $\alpha$ -GlcNAc and  $\alpha$ -celotriose antibodies, respectively, but relatively normal levels of IgA anti- $\alpha$ -Rha antibodies and IgG and IgM antibodies. When changes in antibody level do occur they are frequently gradual and continue over several weeks (eg, serum #1962; IgA anti-celotriose), yet can also be sudden, eg, serum #1972; IgM anti-L- $\alpha$ -Rha, which abruptly increases between week four and five and then again slowly returns to its basic level.



**Figure 1** Relative antibodies binding levels to a set of 34 glycans displayed on a glycans array (see Table 1). Antibodies are from IgG pool of ~10 000 healthy human. Binding levels measured in relative fluorescent units (RFU). The most dominate structures are  $\alpha$ -GlcNAc (glycan 19),  $\alpha$ -L-Rha (glycan 23),  $\beta$ -GlcNAc (glycan 20), Gal( $\alpha$ 1,3)Gal( $\beta$ 1,4)GlcNAc (glycan 34),  $\beta$ 4-linked oligosaccharides of glucose (glycans 14,15,16),  $\alpha$ -Gal (glycan 1) and GlcNAc( $\beta$ 1,4)GlcNAc( $\beta$ ) (glycan 22). Those glycan fragments are common in bacteria, fungi, and parasite cells. Adopted from reference 22.



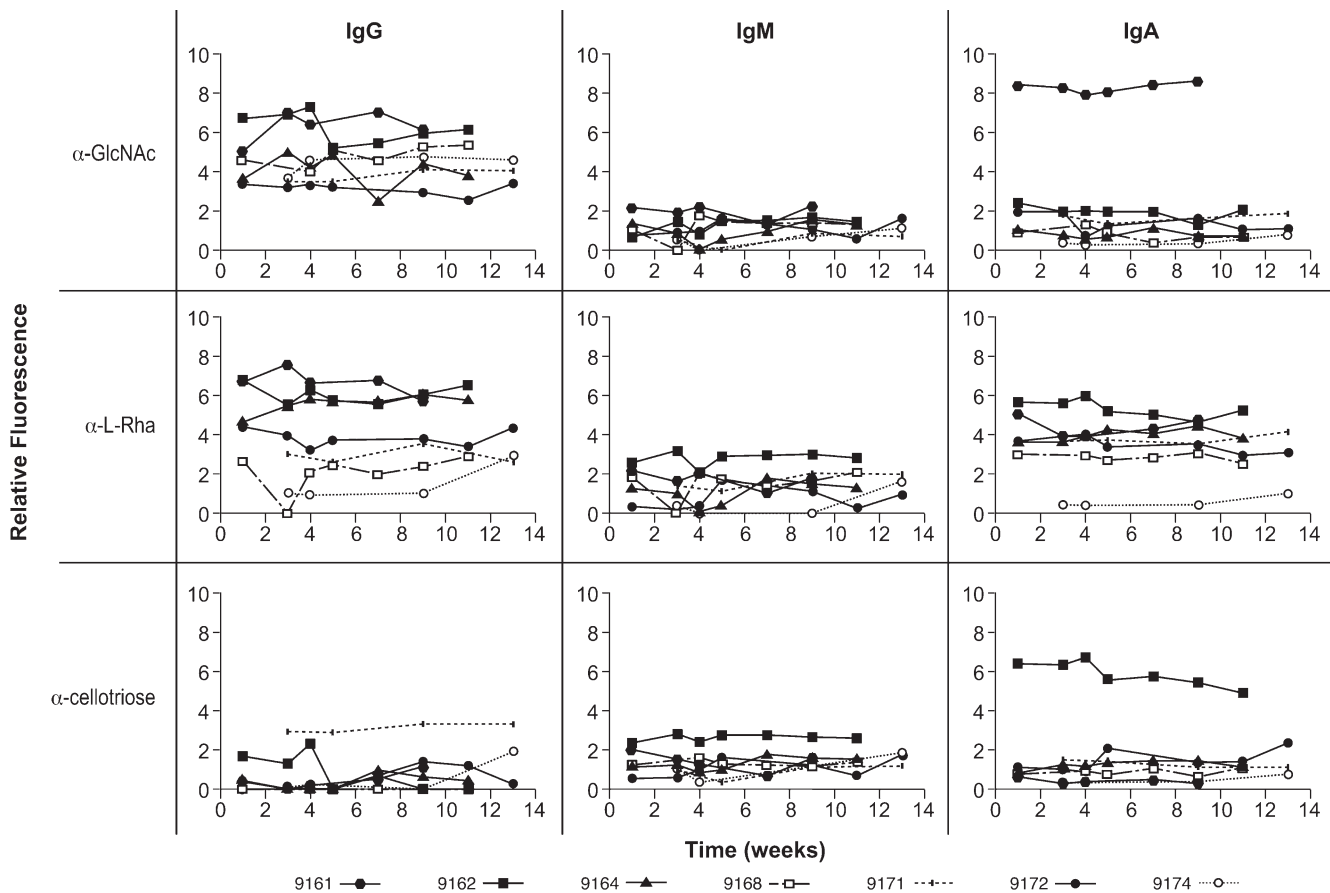
**Figure 2** Distribution of anti-glycan antibodies (IgG, IgA and IgM) levels in 200 sera from apparently healthy blood donors to seven mono- and oligosaccharides. Anti-glycan antibodies were measured as previously described<sup>22</sup> with the following modification: instead of using biotinylated protein A for detection of bound Ig we have used biotinylated goat anti-human Ig antibody, of IgG, IgA and IgM subtypes, for IgG, IgA and IgM determination.

### Anti-glycans antibodies in multiple sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system, the etiology and pathogenesis of which remain largely elusive. Studies in patients with MS and its animal model have suggested that the disease is of autoimmune nature, with a primary T-cell-driven aberrant immune response, but with clear contributions of antigen-driven B-cell responses. The most common form of MS is the relapsing – remitting form (RRMS), in which episodes of acute worsening of neurological function (relapses) are followed by partial or complete recovery periods (remissions) free of disease progression. A diagnostic or prognostic biomarker implicating a simple serological test for the definite confirmation

of MS, and the level of risk in individuals presenting a primary acute demyelinating event would be of utmost clinical importance. There have been numerous attempts to identify antibodies that will serve as biomarkers for MS, of them, notable number of anti glycan antibodies. There have been contradicting reports regarding anti-galactocerebroside IgG antibodies as specific to MS two decades ago, however, recently antibody responses against galactocerebroside were demonstrated in 40% of MS versus 0% in healthy controls, and immunoaffinity purified anti-galactocerebroside IgGs from human serum bind to cultured human oligodendrocytes, indicating that the ELISA detects a biologically relevant epitope.<sup>44</sup> Recently the importance of antibodies recognizing glucose based structures in RRMS patients was emphasized. Anti-N-glycosylated peptide antibodies were reported to be specific for RRMS patients, and aberrant N-glycosylation was suggested as fundamental determinant of auto-antibodies recognition in MS. IgM antibodies to N-glycosylated peptide were found in 21% of 250 RRMS patients, in 27% of 25 patients with other inflammatory neurological diseases, as well as in 6% of 166 normal blood donors.<sup>45,46</sup>

As a result of systematic screening using the GlycoChip<sup>®</sup> glycan array, anti-Glc(α1,4)Glc(α) IgM antibodies (anti-GAGA4) were found to exist in significantly ( $P < 0.0001$ ) elevated levels in MS patients ( $n = 62$ ) in comparison to other neurological diseases (OND,  $n = 48$ ) patients<sup>37</sup> (see Figure 4a and b). Anti-GAGA4 levels enabled to differentiate between RRMS and OND patients with 57% sensitivity, 85% specificity.<sup>37</sup> Anti-GAGA4 antibodies were found to have predictive value for identifying patients that will become RRMS at time of first acute neurological event. Sera samples that were taken from patients at first acute neurological event and kept frozen were tested retrospectively. The study included patients that were followed up for at least four years and have clinically confirmed diagnosis as RRMS and control group of patients who were suspected with MS but eventually diagnosed as other neurological diseases (OND). The level of anti-GAGA4 antibodies was found significantly higher ( $P = 0.005$ ) in patients clinically diagnosed with RRMS than in patients who were suspected as MS but eventually diagnosed with OND. Based on antibodies levels it was possible to differentiate between patients that were diagnosed later as RRMS and patients that were diagnosed later as OND, with 36% sensitivity, 91% specificity, 80% positive predictive value and 58.8% negative predictive value.<sup>38</sup> Anti-GAGA4 antibodies maybe a specific and simple diagnosis and prognosis tool for RRMS patients already at first acute neurological event.



**Figure 3** Temporal stability of anti-glycan antibodies (IgG, IgM and IgA types) levels in seven apparently healthy individual (serum numbers 9161, 9162, 9164, 9168, 9171, 9172, 9174) measured over 13 weeks. All sera samples were analysed stimulatingly.

### Anti-glycans antibodies in inflammatory bowel disease

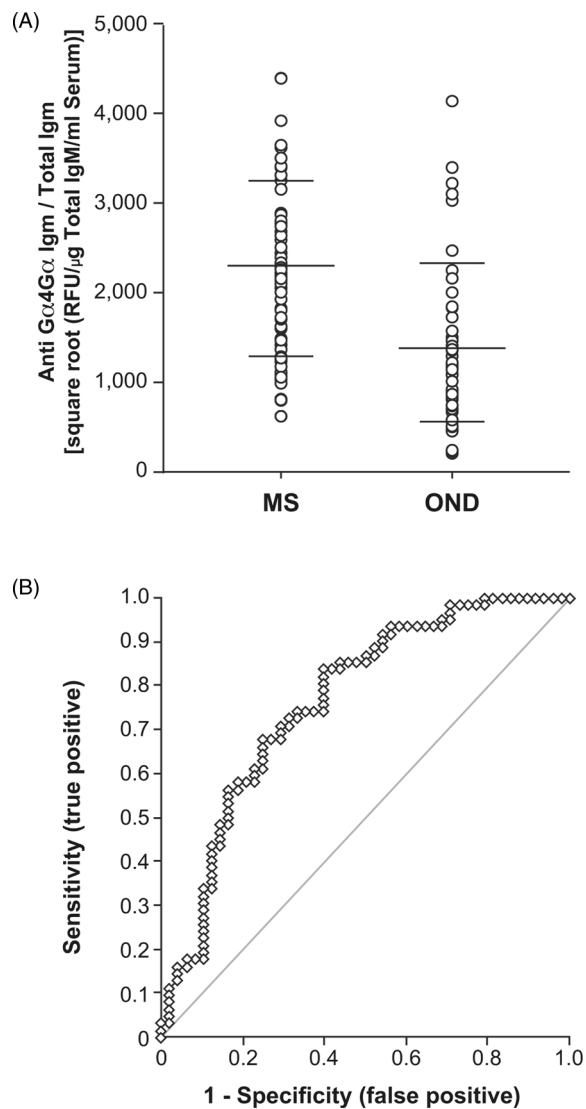
Inflammatory bowel disease (IBD) is a chronic intestinal disorder of unknown etiology comprising two major types: Crohn's disease (CD) and ulcerative colitis (UC). The diagnosis of IBD and the differentiation between UC and CD are established by the combination of clinical, laboratory, radiological, endoscopic, histopathologic and serological characteristics. However, when a definite diagnosis cannot be established, as is true in 10–17% of colitis patients, a diagnosis of indeterminate colitis (IC) is designated.

In addition to diagnosing IBD, serological markers may be used to discriminate between CD and UC, assess disease activity and progression, predict response to treatment and to stratify disease severity. The major serological markers for IBD in commercial use are anti-perineutrophil cytoplasmic antibodies (pANCA) and anti-*Sacharomyces cerevisiae* antibodies (ASCA). ASCA are directed against oligomannosidic residues on the polysaccharide mannan in the cell

walls of the yeast *S. cerevisiae* and have a prevalence of 48–69% among CD patients and 15% among UC patients.<sup>47</sup> ASCA is associated with severe small-bowel disease rather than colonic disease and with penetrating CD. ASCA can be detected in CD patients years before diagnosis of CD. ASCA was positive in 32% of serum samples from apparently healthy recruits to the Israeli Defense Forces, who were later diagnosed as having CD.<sup>48</sup>

### Anti-glycan antibodies improve diagnosis of Crohn's disease

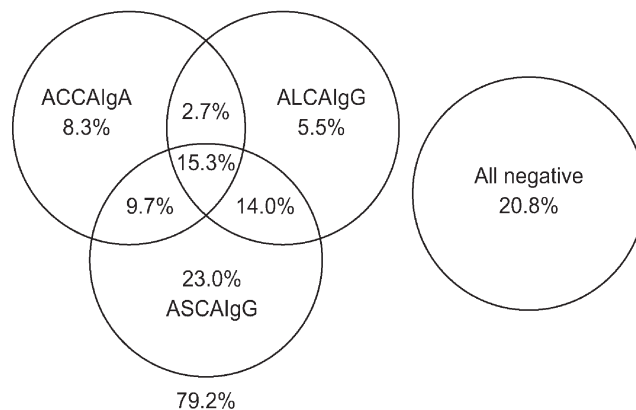
Systematic screening for anti glycan antibodies in IBD using glycan array have lead to the discovery of novel anti-glycan antibodies that may be associated with inflammatory bowel disease. Anti-laminaribioside (Glc(β1,3)Glc(β)) carbohydrate IgG antibodies (ALCA), anti-mannobioside (Man(α1,3)Man(α)) carbohydrate IgG antibodies (AMCA), and anti-chitobioside (GlcNAc(β1,4)GlcNAc(β)) carbohydrate IgA antibodies (ACCA) had the highest discriminative capability



**Figure 4** Anti-Glc(α1,4)Glc(α) IgM in RRMS and OND patients. (A) Distribution of antibodies in MS ( $n = 62$ , 16 PPMS and 46 RRMS patients), and OND ( $n = 48$ ) groups. Box plot describing distribution of anti-Glc(α1,4)Glc(α) (Gα4Gα) antibodies (square root) normalized according to total IgM levels (square root). The long bar represents mean values, shorter bars above and below mean indicate mean plus and minus standard deviation. (B) Receiver operating characteristics (ROC) curve for different anti-Gα4Gα IgM cutoff values (RFU/Ag total IgM/mL serum)<sup>0.5</sup> for differentiating between MS ( $n = 62$ , 16 PPMS and 46 RRMS patients), and OND ( $n = 48$ ) groups, when MS is the outcome of interest. Area under the curve 0.765 (95% CI 0.673–0.865). Adopted from reference 37.

between Crohn's disease and ulcerative colitis.<sup>39,40</sup> Importantly, 44% of anti-Sacharomyces cerevisiae antibody (ASCA) negative Crohn's disease patients were positive for anti-laminaribioside or anti-chitobioside.<sup>39</sup> (see Figure 5). Larger scale studies, using immuno assay developed for detection of ALCA, ACCA and gASCA, have shown ALCA, ACCA and gASCA levels were

CD Cohort ( $n = 72$ )



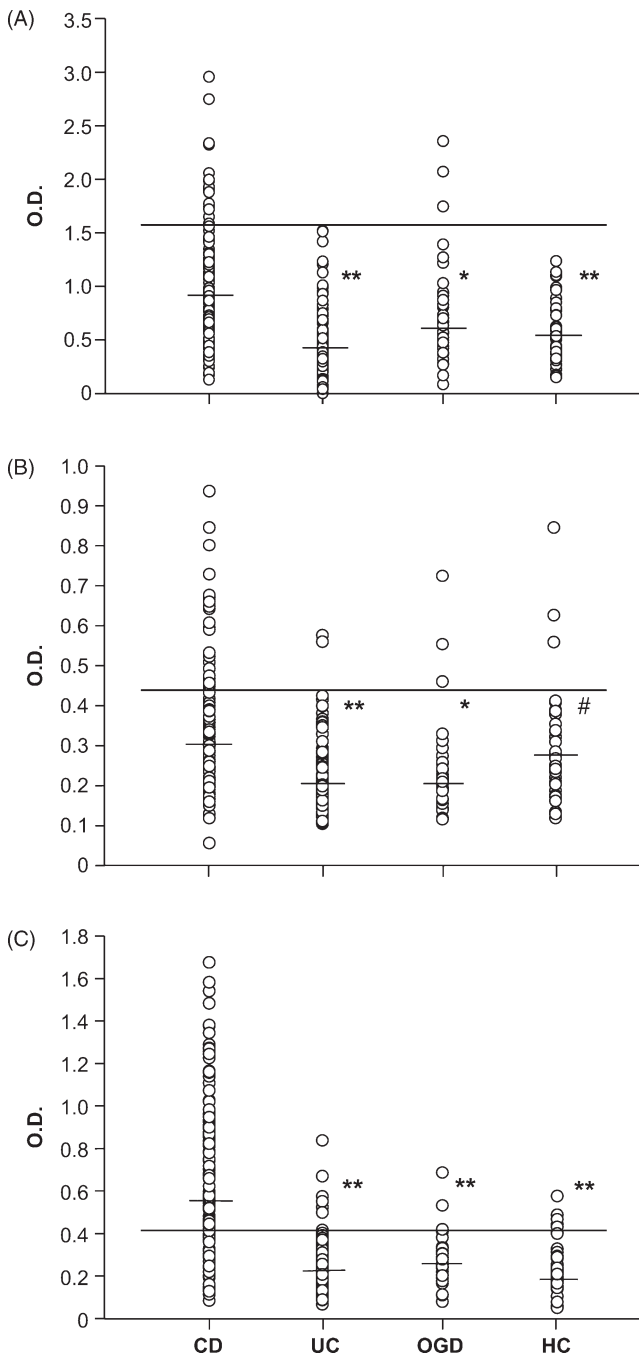
**Figure 5** Relationship between ALCA, ACCA and ASCA in the CD cohort by presence versus absence. Percentage of the CD patient cohort ( $n = 72$ ) that is positive for each marker, any combination of two markers, and all markers is shown. ALCA and ACCA were determined by the glycan array and ASCA by the commercial ASCA kit (Inova Diagnostics), as described in the materials and methods section. A total of 44% of ASCA-negative CD patients were positive for ALCA or ACCA. Adopted from reference 39.

significantly higher in CD patients in comparison not only to UC patients, but to a panel of patients with other gastro diseases as well (see Figure 6). In patients with inflammatory bowel disease that were positive for antibodies against either ALCA, ACCA or ASCA, the diagnosis of Crohn's disease was suggested with a sensitivity of 77.4% and specificity of 90.6%. Having at least two of these antibodies increased the specificity to 99.1%.<sup>41</sup>

#### Anti-glycan antibodies predict complicated disease course and surgery in Crohn's disease

Crohn's disease (CD) presents heterogeneously and may often lead to complications needing surgery, identification of those patients at early stage could lead to better treatment and disease management. Several serological markers (ASCA, pANCA, anti-OmpC, anti-I2) have been identified in IBD and are associated with distinct clinical phenotypes.<sup>49</sup> We tested if a panel of anti-glycan antibodies could predict complicated disease course (as fistulas or strictures) or the need for surgery in Crohn's disease.

In a study in a Israeli cohort of CD patients ( $n = 124$ ) we have found that in addition to their value in improving diagnosis of CD patients, higher levels of antibodies against laminaribioside or mannan in CD patients, were significantly associated with a more complicated disease behavior of stricturing and fistulizing, as well as association to smaller intestinal disease.<sup>41</sup>



**Figure 6** The distribution of ALCA, ACCA and gASCA in IBD patients and controls. Serum samples from 124 CD, 106 UC and 61 non-IBD gastrointestinal disease patients (OGD) and 40 healthy controls were screened for the presence of (a) ALCA, (b) ACCA and (c) gASCA by immuno assay as described in the materials and methods section. The median values for each group are indicated by the short horizontal lines. The crossing line indicates the cut-off value used for defining positive and negative results. OGD group included 27 celiac disease, 20 irritable bowel syndrome (IBS) patients and 14 patients with colonic polyps, diverticular disease, pseudomembranous colitis, helicobacter pylori gastritis, pancreatitis, and hemorrhoids. Adopted from reference 41. \* $P < 0.05$  versus CD; \*\* $P < 0.0001$  versus CD; # $P = 0.004$  versus UC.

Results of a larger scaled study<sup>50</sup> in a Belgium cohort of CD patients ( $n = 755$ ) (mean age 42.3 years, 58% female) that tested a panel of anti glycan antibodies (ALCA, ACCA, AMCA and gASCA) as well anti-bacterial outer membrane porin antibodies (Omp<sup>+</sup>), show that ACCA, AMCA and Omp<sup>+</sup> were independently associated with need for surgery (all  $P < 0.01$ ). gASCA also showed a trend for association with the need for surgery ( $P = 0.06$ ) and was independently associated with a non-inflammatory behaviour. Assessing the data from a clinical perspective, a complicated disease course (strictures or fistulas) needing surgery was independently associated with gASCA ( $P = 0.002$ ), ACCA ( $P = 0.011$ ), Omp<sup>+</sup> ( $P = 0.008$ ), and ileal ( $P = 0.043$ ) as well as anal ( $P = 0.002$ ) involvement.

Combining the different marker scores resulted in 86 patients negative for all five markers (group A), 490 positive for one to three markers (group B) and the remaining 179 positive for more than three markers (group C). A more complicated disease course (presence of strictures or fistulas) was noted with an increasing score (from 35% to 60% to 83% respectively; group A versus B odds ratio 2.80 (CI 95% 1.73–4.53); group A versus C odds ratio 9.10 (CI 95% 5.01–16.55)). Very similar findings were found using the need for surgery as outcome (from 33% to 51% to 79% respectively; group A versus B OR 2.19 (1.35–3.56); group A versus C OR 7.95 (4.46–14.17)). These results illustrate that serological markers directed against glycan-epitopes (gASCA, ACCA, AMCA) and the other membrane porins (Omp<sup>+</sup>) are all associated with severe and complicated Crohn's disease and with the need for surgery.

#### Anti-glycan antibodies and innate immunity system

Laminaribioside, chitobioside and mannobioside are not auto-antigens in humans; however they are common structures in glycocalyx of pathogenic yeast and bacteria. Laminaribioside is the building block of laminarin, a polysaccharide of the  $\beta 1,3$ -glucan family. Beta-1-3 glucans may be found in the cell walls of saprophytic and pathogenic fungi and yeast, including *S. cerevisiae*, as well as in food (oats) and algae. Chitobioside is a component of chitin, polymer of  $\beta 1,4$  linked *N*-acetyl-D-glucosamine, a major component of the insect cuticle as well as the cell walls of infectious pathogens such as bacteria and yeast. Mannobioside is a component of mannan from pathogenic fungi and yeast, including *S. cerevisiae*.<sup>47</sup> Mannan,  $\beta 1,3$  glucans and chitin or fragments thereof, may bind to specific receptors on neutrophils, macrophages and NK cells, thereby stimulating cell proliferation, phagocytosis and cytokine secretion. In a study including 755 CD

patients, significant association was found between mutations in innate immune genes that are known to be related to CD (CARD15 and TLR4) and levels of gASCA, ALCA and ACCA.<sup>51</sup> Compared with wild type, CD patients with one or more CARD15 variants were more gASCA positive (49.4% versus 63.7%,  $P < 0.0001$ ), and had higher gASCA titres (61.9 U versus 83.7 U,  $P < 0.0001$ ). A similar finding was observed for ALCA (32.7% versus 45.9%,  $P < 0.0001$ ; 41 U versus 46.8 U,  $P = 0.003$ , respectively). Furthermore, the prevalence of ASCA and ALCA increased with the number of variants, suggesting a gene dosage effect: ASCA prevalence increased from 49.6% to 61.2% and 71% ( $P < 0.0001$ ), and ALCA prevalence from 32.7% to 45.3% and 47.5% ( $P = 0.002$ ), for 0, 1 and 2 CARD15 variants, respectively. For TLR4, an opposite effect was seen, as CD patients carrying a variant less frequently expressed ACCA (22.5%) compared with wild type patients (37.2%,  $P = 0.003$ ). ACCA prevalence in CD decreased from 37.2% to 24.5% and 0% ( $P = 0.001$ ) for 0, 1 and 2 variants, respectively. Thus,  $\beta$ 1,3 glucans and chitin have the potential to modulate the immune system, specifically its innate arm while the finding of antibodies against these components that are specifically associated with CD suggests a link to adaptive immunity.

ALCA, ACCA and AMCA are novel serological markers associated with inflammatory bowel disease and especially with Crohn's disease. Their combined use with ASCA, contributes to the diagnosis of IBD. Those markers are all associated with severe and complicated Crohn's disease and with the need for surgery. Furthermore, a panel of serological markers may define Crohn's disease patients with different disease outcome.

## Conclusions

A diverse repertoire of anti-glycan antibodies of all types exists in human sera. The development of glycan array technologies such as the GlycoChip<sup>®</sup> enables to determine the anti glycan antibodies profile (AGAP) in sera in a quick and robust way. These profiles were shown as quite stable over a time frame of months. Using the GlycoChip<sup>®</sup> for measurement of the AGAP in populations of patients in MS and CD, and comparing them to the profiles of control groups, has lead us to the discovery of anti-glycan antibodies specific to MS or CD. Anti-Glc( $\alpha$ 1,4)Glc( $\alpha$ ) IgM were found to be specific for MS. Anti-Glc( $\alpha$ 1,4)Glc( $\alpha$ ) IgM were found in MS patients at the early stage of the disease, at the time of first acute neurological event, their existence predicts high risk for conversion to RRMS

within 24 months. A different panel, of anti-glycan antibodies (ALCA, ACCA and AMCA) was found to be specific for CD. Those antibodies were found to be associated with a more complicated disease course and with a higher risk for surgery as well as innate immunity gene mutation. We have shown that anti-glycan antibodies specific to inflammatory and immune modulated diseases can be discovered using the GlycoChip<sup>®</sup> technology. Large scale validation studies have shown that those anti-glycan antibodies are valuable not only for diagnosis, but for prediction of more complicated and severe disease course in CD and more active disease in MS.

AGAP is not limited to inflammatory or autoimmune diseases and it holds promise for other areas. Lately it was reported that antibodies to glycans associated to breast cancer were discovered using glycan array<sup>52</sup> those antibodies may be valuable in non-invasive screening for neoplasia (early detection for high-risk women), as well as prediction of treatment efficacy. Another potential use of AGAP may be related to vaccine development, using glycan array, neutralizing antibodies elicited by SARS vaccine was found to have an undesired autoimmune reactivity to a glycan fragment common in humans.<sup>53</sup> AGAP is a new and promising approach for discovery and development of anti-glycan antibodies as biomarkers for diagnosis and prognosis.

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