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Non-invasive biomarkers in chronic inflammatory bowel disease: State of the art

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Abstract

The need to improve the diagnosis and classification of chronic Inflammatory Bowel Disease (IBD) on the one hand, the monitoring and prognostic prediction on the other hand, have prompted scientific efforts to improve the characterization of the already-known markers and to search for other potential effective parameters. Consequently, the predominant target of polynuclear neutrophil Anti-Cytoplasma (ANCA) in ulcerative colitis (DNA-bound lactoferrin) and exocrine anti-pancreatic antibodies (Ab) in Crohn's Disease (CD)) (GP2 and CUZD1) have recently been identified. Moreover, new markers have shown their potential clinical utility during IBD. Most of them have been associated with CD, such as anti-glycans which include in addition to classic ASCA, other recently described markers (Anti-Laminaribioside (ALCA), Anti-Chitobioside (ACCA), antimannobioside (AMCA), anti-laminarin (anti-L) and anti-chitin (anti-C)). Other anti-microbial antigens Ab (anti-OmpC, anti-Cbir1 and anti-I2 sequence Ab) have been also reported and associated with certain clinical phenotypes of CD. Regarding inflammatory markers, serum C-reactive protein, calprotectin and faecal lactoferrin, which are considered to be simple and very well studied markers, have shown their reliability in the differentiation between IBD and irritable bowel syndrome, the classification of the degree of intestinal inflammation and the follow-up under treatment. Finally, non-coding RNAs, the more recently described biomarkers, seem to be detectable in blood and useful in enhancing diagnosis, classification and monitoring disease activity.

Introduction

Chronic Inflammatory Bowel Disease (IBD) is considered as a heterogeneous group of chronic inflammatory conditions affecting the gastrointestinal tract [1]. Their diagnosis is conventionally based on a set of clinical, radiological, endoscopic and histological arguments. Currently, biomarkers could be useful as helpful clinical tools for diagnosis and for prediction of disease course and therapeutic response [2-5]. The reliability of a biomarker depends on its effectiveness in differentiating IBD from other non-IBD diseases, having a similar clinical presentation and in discriminating between Crohn's Disease (CD) and Ulcerative Colitis (UC), the 2 main forms of IBD. The biomarker usefulness also depends on its prognostic prediction and its role in monitoring disease activity, including under treatment [6].

Among the various known serological markers, only anti-neutrophil cytoplasmic antibodies (Ab) (ANCA) and *anti-Saccharomyces cerevisiae* Ab (ASCA) have demonstrated diagnostic utility. Their simultaneous research improves the specificity in the distinction between MC and UC. However, the interest of ASCA and ANCA is limited, in particular because of their moderate sensitivities. Moreover, these auto-Abs are not predictive of disease activity, and are of no interest in monitoring treatment [1,4,6-8]. More recently, non-coding RNAs (ncRNAs) have attracted a lot of interest in the context of IBD. Prelimi-

nary data reported emerging roles of some of these markers in the diagnosis, treatment and prognosis of IBD and its associated-colorectal cancer. The need to improve the diagnostic and classification tools, on the one hand, and the monitoring and prognostic prediction (activity, course, response to treatment) of IBD on the other hand, has led efforts to better characterize the already-known markers and to look for new promising parameters [5,6,9].

Classic IBD markers

Currently, the conventionnel markers for IBD diagnosis in routine practice remains ASCA and ANCA. Other previously-described markers are less frequently associated with IBD: they are mainly exocrine anti-pancreatic antibodies (APE) (in CD); but also anti-goblet cells of the intestine (ACCI) (in UC) which is less described and studied.

ANCA (Anti-Neutrophil Cytoplasmic Antibodies)

During IBD, ANCA seem to target a nuclear and non-cytoplasmic component of PNNs. In this context, these Ab are called atypical p-ANCAs of the "Nuclear Associated Neutrophil Antibodies" (NANA) or x-ANCA type. They are specific markers of UC with a specificity greater than 88% [1]. Thus, serum levels of ANCA could be a helpful tool for the diagnosis of UC [10]. Furthermore, monitoring of ANCA-IgG levels could predict disease course and may guide treatment of UC [11]. In particular, in patients with severe UC, pANCA may be useful in determining the clinical response to infliximab [12].

The screening for NANA (IgG) is carried out first by Indirect Immunofluorescence (IIF) on human PNNs fixed with ethanol and then by IIF on human PNNs fixed with formalin and methanol. The initial p-ANCA pattern, lost on the formalin slide, could be found on the methanol slide. Moreover, this p-ANCA aspect is atypical compared to ANCA directed against myeloperoxidase (MPO-ANCA), with a thinner and less regular border surrounding the nucleus of PNNs (Figure 1) [1].

For a long period of time, the exact antigenic target of NANA has remained unknown. Several studies proposing nuclear components of PNNs as potential or possible targets for these auto-Abs have been ruled out. Lactoferrin has been suggested as a target of NANA during UC. However, the detection of this molecule was impossible using Western Blot or monospecific ELISA (purified lactoferrin bound to the solid phase) techniques. Recently, some authors have shown that it is exactly the lactoferrin bound to DNA, detected by IIF performed on granulocytes treated with saline solution and reconstituted with human lactoferrin "LFR granulocytes", which is the major target of NANA during of UC (72% of cases) [13].

A pathophysiological role has been suggested for these DNA-bound anti-lactoferrin Ab. They seem to be able to bind to components of chromatin and to PNN proteins, involved in the composition of extracellular traps "Neutrophil Extracellular Traps" (NETs). Granulocytes, in general, and NETs in particular, are considered as cornerstones of the innate defense against microbes (GRAM positive and negative bacteria), especially in barriers such as the intestinal mucosa. Therefore, the auto-Abs directed against the components of nets could affect this antimicrobial defense at this barrier, which suggests the link between microbial infection and the onset of autoimmunity during IBD [13].

ASCA (anti-Saccharomyces cerevisiae Ab)

Described in the late 1980s, these Ab react with brewer's and bread yeast, *Saccharomyces cerevisiae*. Since then, they have been known as a specific marker for CD within adults as well as children with a specificity above 90%. In clinical practice, serum ASCA levels was found to be the most accurate serological marker for the differential diagnosis of CD [14]. In addition, combined tests of serum ANCA-IgG, ASCA-IgG, and ASCA-IgA levels may help to distinguish UC from CD [11,15].

The antigenic target recognized by these Abs is found in the soluble extract of the yeast wall. This is phosphopeptidomannan, commonly referred to as "Mannan" (GP of 200 kDa). The antigenic determinants are more precisely trimannoside epitopes. ASCA are detected by IIF on a culture of *S. cerevisiae*, with a fluorescent pattern of the wall of yeasts (Figure 2).

Besides, ASCA can be detected by Elisa or by immunodot. These two techniques use antigens extracted from boiled or disrupted yeast or phosphopeptidomannans purified from the wall of yeasts [1]. Recently, "gASCA", an improved ASCA test, has been described, which is based on the covalent immobilization of purified "mannan" polysaccharides, and has proved to be efficient in comparison with the conventional ASCA test [16].

On the other hand, recent researches have revealed promising new markers of the anti-glycans family, to which ASCA belongs. Most of these recent parameters are associated with CD (see below "Abs directed against microbial antigens") [6].

Anti- Exocrine Pancreas antibodies (AEP)

Described for the first time in 1987 in patients with CD, AEP (IgG) are detected by IIF on sections of primate or human pancreas. Two types of fluorescence patterns can be distinguished: the first is an extracellular pattern in drops located in the lumen of the pancreatic acinis, while the 2nd is an intracellular reticulo-granular pattern detected within the acinis (Figure 3).

It has been shown that AEP have an excellent positive predictive value for IBD (99%). Their excellent specificity for CD was re-discussed after the study conducted by Joossens et *al*., where authors showed a prevalence of 32% of AEP in CD but also a positivity of 23% in UC and 22% in healthy relatives of patients [1]. Recently, molecular targets for APEs have been identified [17-19]:

* Fluorescence pattern in drops in the lumen of acini is related to the major target of APE during IBD: "the zymogen granule glycoprotein 2 (GP2)".This target is also present on the surface of the "Microfold" M cells of Peyer's patches and appears to play an immunomodulatory role in the intestine.

* The intracellular reticulo-granular fluorescence pattern within acinis is mainly due to Ab directed against the target "CUB and zona pellucida-like domains containing protein 1 (CUZD1)". GP2 and CUZD1 belong to the family of innate immunity proteins. It seems that these two molecules are involved in maintaining the balance between tolerance to commensal bacteria and defense against pathogens in the intestine [17].

The characterization of these targets is carried out by IIF on transfected HEK 293 cells (Figure 4) or by ELISA.

The anti-GP2 and anti-CUZD1 Ab have been described mainly during CD (Table 1). Most studies focused on determining the clinical relevance of anti-GP2. Serum level of this Ab is more detected in patients with CD than in UC [6]. The detection of anti-GP2, in combination or not with ASCA, allows a phenotypic classification of CD patients. In fact, anti-GP2 can be used to evaluate the clinical severity, especially with early onset, pouchitis and pouch surgery [20].

Studies related to Anti-CUZD1 remain very limited. However, IgA isotype of this marker appears to be associated with complications in CD.

Anti- Caliciform Cells of the Intestine (ACCI)

More than 50 years ago, these Ab were described as pathognomonic markers of UC but with a low prevalence of 28%. They are detected by IIF. The substrate of choice is fetal primate intestinal tissue. The presence of these Ab is responsible for an indistinct borderline "woolly" fluorescence (Figure 5).

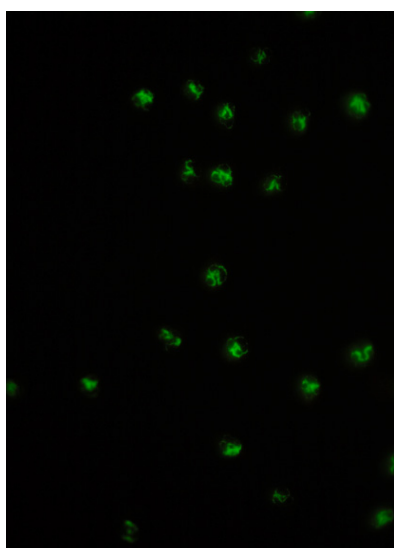

Figure 1: Atypical p-ANCA pattern (NANA «Nuclear Associated Neutrophil Antibodies») in IIF on PNN fixed with ethanol.

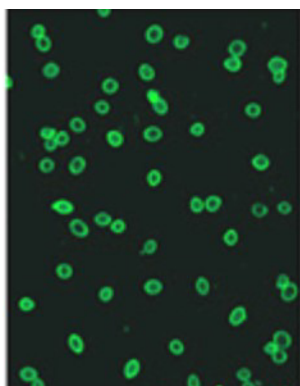

Figure 2: ASCA pattern in IIF on S. cerevisiae culture.

Markers directed against microbial antigens

The spectrum of antibodies directed against different microbial antigens which are described as associated with IBD continues to expand rapidly. Most of these Abs are associated with CD, such as the family of anti-glycans, which in addition to the classic ASCA, include other recently described markers: anti-laminaribioside Ab (ALCA), anti-chitobioside (ACCA), anti-mannobioside (AMCA), anti-laminarin (anti-L) and anti-chitin

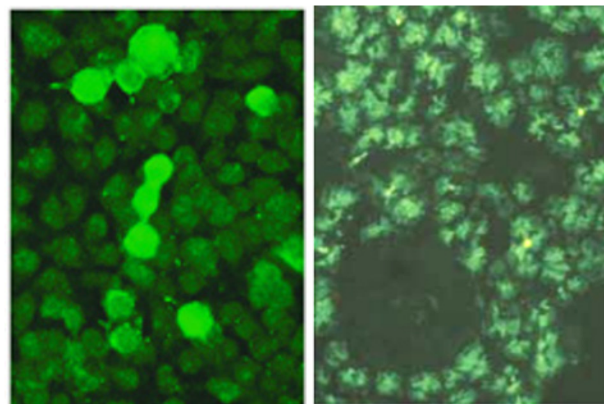

Figure 3: Different patterns of Ab anti-exocrin pancreas (AEP) in IIF on primate's pancreas.

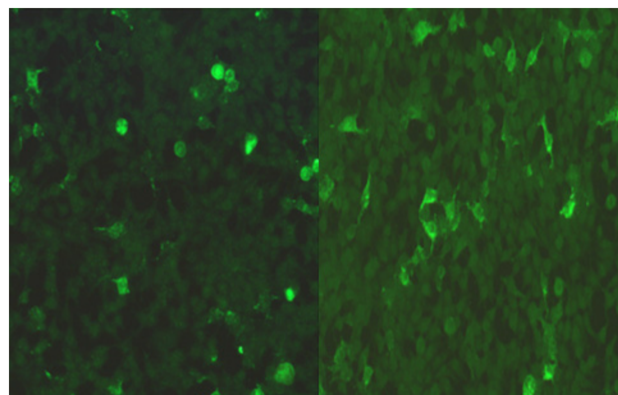

Figure 4: Immunofluorescence patterns on transfected HEK 293 cells of the two types of anti-exocrin pancreas (AEP) antibodies: anti-GP2 (in the left) and anti-CUZD1 (in the right).

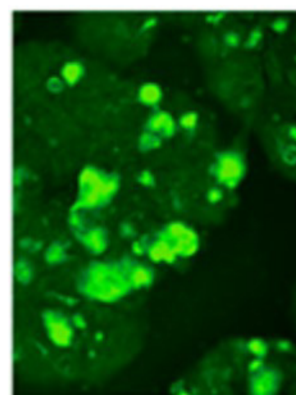

Figure 5: Pattern of Ab anti-caliciform intestinal cells (ACCI) in IIF on primate intestine.

(anti-C) (Table 1). Despite of the lack of sensitivity of these biomarkers for the diagnosis of IBD, they are capable of identifying a small group of patients with CD seronegative for the conventional markers (such as ASCA). AMCAs, ALCAs and ACCAs can be used for the differentiation between CD and UC [17]. The specificity of anti-L and anti-C for CD diagnosis is relatively high with a low sensitivity. Therefore, the combination of these Abs with ANCA and ASCA detection may be more helpful in the differentiation of CD from UC [18-22]. Moreover, the new anti-glycans seem to be correlated with the complicated forms of CD and are associated with a severe course and the need for surgical treatment [6].

Other anti-microbial antigens Ab have been reported during

IBD: anti-OmpC "anti-Escherichia coli Outer membrane porin C", anti-Cbir1 flagellin and Ab "anti-I2 Pseudomonas fluorescens sequence" were particularly associated with certain clinical phenotypes of CD (stenosing and penetrating forms) [6,23]. More recently, Ab against 2 other flagellins (A4-Fla2, Fla-X) have been identified and appear to be associated with complications [24,25]. However, given their low sensitivity, the role of these Abs in the diagnosis and prognostic evaluation during IBD remains a controversial issue.

Table 1: Characteristics and prevalence of the new serological markers (anti-exocrine pancreas and anti-microbial antigen antibodies) of inflammatory bowel disease [6,20,24,25].

Biomarker	Antigenic target	Isotype	Technique of detection	Prevalence (%)				Ref
				CD	UC	Other GI disorders	Unaffected controls	
Anti-pancreas exocrin								
anti-GP2	Pancreatic major glycoprotein GP2 of the zymogen granule membrane	IgA	IIF ELISA	1-25	0-13	4 29-38 celiac disease	0-4	26-33.
anti-GP2		IgG	IIF ELISA	10-44	1-22	0–1 0-19 celiac disease	0-8	26-34.
anti-GP2		IgA and/or IgG	IIF ELISA	21-45	2-19	4	1-8	26-30, 32, 33.
anti-CUZD1	CUB and zona pellucida-like domains containing protein 1	IgA	IIF	12.1-16.1	2.9-6.2	---------	0	15, 35-37.
anti-CUZD1		IgG	IIF	16.3-17.9	4.4-6.7	---------	0	
anti-CUZD1		IgA and/or IgG	IIF	21-26	5.9-9	---------	0	
Antibodies anti-microbial antigen								
gASCA	Purified carbohydrate PPM epitopes from *S. cerevisiae* wall	IgA and/or IgG	ELISA	0-69	0-14	1-23 11-22 celiac disease	0-15	21, 22, 38-45.
ACCA	Chitobioside (GlcNAc (β1, 4) GlcNAc (β))	IgA	ELISA	8-52	0-45	3-35 22 celiac disease	2-33	21, 22, 38-40, 42-46.
ALCA	Laminaribioside (Glc (β1, 3) Glc (β))	IgG	ELISA	8–76	0-22	1-21 0-7 celiac disease	0-23	21, 22, 38-47.
AMCA	Mannobioside (Man (α, 3) Manα	IgG	ELISA	12-67	0-36	3-27	0-33	22, 38-40, 42-46.
Anti-C	Chitin (GlcNAc (β1, 4)) n	IgA	ELISA	10-25	2-15	7-23	2	22, 39, 42.
Ani-L	Laminarin (Glc (β1, 3)) 3n (Glc (β1, 6)) n)	IgA	ELISA	11-26	3-15	4-11	2	22, 39, 42.
≥1 anti-glycan				59-78	28-48	21-50	21-23	22, 24, 39, 40, 42.
Anti-OmpC	Escherichia coli outer membrane porin C	IgA	ELISA	24-55	2-24	5-11	5-20	37,48-62.
Anti-I2	I2 Pseudomonas fluorescens sequence	IgA	ELISA	38-60	2-10	19	5–15	48-51, 54, 55, 60, 63, 64.
Anti-Cbir1	Cbir1 bacteria flagellin	IgG	ELISA	50-56	6-36	14	8	50, 53, 55, 57, 65, 66.

CD: Crohn's disease; UC: ulcerative colitis; GI: gastro-intestinal, IIF : Indirect Immunofluorescence; ELISA: Enzyme-Linked Immunosorbent Assay.

Benefits of inflammation markers during IBD

Subjective assessment of disease activity during IBD is often described as unreliable. The objective criteria for measuring inflammation correlate much more with long-term outcome, but depend generally on invasive and expensive procedures such as ileocoloscopy and imaging. Non-invasive, accurate and inexpensive indicators of intestinal inflammation would allow the clinician to better adjust therapies and thus, to improve the control of inflammation [2,7]. Several non-invasive inflammatory markers were tested in blood, stool and other biological fluids (urine). While no marker has been universally adopted, some have been well characterized, and others appear to be very promising.

Serum C-reactive protein "C Reactive Protein (CRP)" and fecal calprotectin (cytosolic protein of neutrophils binding zinc and calcium, easily detected in the stool) are among the best studied, simple and non-invasive biomarkers of inflammation in IBD [2,7,8], their reliability has been described in the differentiation between IBD and irritable bowel syndrome, in the classification of the degree of intestinal inflammation, in the evaluation of the response to treatment, and eventually, in the detection of recurrent inflammation after remission.

Thus, CRP and fecal calprotectin are currently useful in clinical practice for the management of IBD patients [10,67] In addition to these two markers, the detection of lactoferrin in the stool (thermostable protein derived from PNNs that have mi-

grated into the intestinal mucosa), appears to be a promising indicator for monitoring intestinal inflammation [68].

Non-coding RNAs in IBD

NcRNAs, including microRNA (miRNA), long ncRNA (lncRNA) and circular RNAs (circRNAs), have gained a lot of interest last years. Although they account for ~90% of RNAs, these molecules have no protein coding potential and are important regulatory mediators transcribed from the genome. They control gene expression at the RNA level [8,69]. NcRNAs abnormal expression in blood or tissues has been associated with several autoimmune and malignant disorders [8]. Regarding IBD, it seems that the expression level of circulating and tissue ncRNA is different in patients compared to healthy controls. In particular, miRNAs, a short and stable ncRNAs (18-24 nucleotides) involved in the negative regulation of gene expression at the post-transcriptional level by binding the 3'- untranslated region of mRNA (inhibition of translation or degradation of mRNA), seems to be a promising non-invasive biomarker of disease activity in blood [5,8]. Several studies showed that miRNAs may be involved in the mediation of inflammatory responses, intestinal barrier dysfunction and gut microbiota interactions [5,8,70,71]. Most of the recent research in IBD has measured levels of circulating miRNAs in body fluids such as blood or feces, and in homogenized tissue biopsies using techniques like microarray profiling, RT-qPCR, and NGS [72]. Serum concentrations of some miRNAs (such as miR-16, miR-21, and miR-223) are reported to be higher in IBD patients than in healthy controls and levels may differ between CD and UC patients. MiRNAs have been therefore identified as promising diagnostic biomarkers (to differentiate IBD from other non-IBD diseases and UC from CD) and potential therapeutic targets [2,4,5,73,74]. It could potentially be used for disease management in IBD.

Furthermore, LncRNAs, a non-coding RNAs involved in the regulation of various intracellular processes and have a length of more than 200 nucleotides, is also reported to be a potential relatively stable and simply detectable biomarker for IBD diagnosis [75]. LncRNAs have been proven to play important role in IBD pathogenesis, including regulation of the intestinal epithelial barrier, cell apoptosis, and various immune system processes [8]. Preliminary studies have shown that LncRNAs' expression may be different between IBD patients and healthy controls as well as between CD and UC patients [8,76]. The profile of this biomarker in blood needs to be more investigated, to identify new lncRNAs, and to assess their diagnostic value as a non-invasive biomarker in IBD.

Regarding CircRNAs which are considered as microRNA sponges regulating gene expression at the transcriptional or post-transcriptional level, the alteration of their expression in IBD results in intestinal epithelial barrier and immune homeostasis dysregulation [69,77]. Available reports suggest that CircRNAs (ex: circRNA_004662) might be a novel candidate for differentiating CD from UC as well as a promising prognosis marker [69]. On the other hand, circRNA_103516 level in PBMCs was found to be a potential biomarker for diagnosing IBD [78]. Additionally, it seems that some circRNAs may serve as a promising target for the disease therapy [8].

Oncostatin M

Oncostatin M (OSM) is a member of IL-6 cytokine family. A high and consistent expression of this marker in affected mucosa and in blood has been reported in IBD patients [4,8,79]. Serum level is particularly elevated in active IBD patients as well as in unaffected first-degree relatives of IBD patients [8,79]. It has been reported that serum level of OSM could be a diagnostic biomarker of IBD [4,8,79]. However, it seems that this circulating level could not predict the disease outcome and treatment responsiveness, in contrary with colonic OSM level [80].

Conclusion

So far, no national, European or international recommendation exists concerning the routine detection of Ab (including ANCA and ASCA) for the diagnosis and prognostic prediction of IBD. However, several recent studies have suggested the potential clinical utility of some new markers in IBD, which can be detected in serum, (anti-GP2, new anti-glycans...) as well as in the stool (fecal calprotectin and lactoferrin) at different times in the natural history of the disease. Due to the variation in the results from one study to another (sample size, inclusion criteria, techniques and methodology used, etc.), more prospective data are required to better evaluate the behavior of these markers in relation to the disease course, in particular during the treatment. Non-coding RNAs offer a promising window of opportunity to identify potential non invasive diagnostic blood markers and treatment targets for IBD and its associated-colorectal cancer.

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