

00 Genetics, immunology and biomarkers in clinical practice: do they assist in clinical management?

P. L. LAKATOS

INTRODUCTION

In recent decades there has been a change in the epidemiology of inflammatory bowel diseases (IBD) in North America, as well as Western, and some Eastern European countries¹⁻³. Both Crohn's disease (CD) and ulcerative colitis (UC) possibly stem from common mechanisms, while the exact aetiology remains unknown⁴.

Nowadays a generally accepted hypothesis is that UC and CD may develop from a dysregulated response of the mucosal immune system towards microbial antigens in genetically predisposed individuals. IBD are identified as multifactorial, polygenic diseases with probable genetic heterogeneity. According to this hypothesis, different genetic backgrounds may explain the various clinical patterns of the disease^{4,5}. In addition to genetic predisposition, various environmental and host factors (e.g. genetic, epithelial, immune, and non-immune) play a major role in the pathogenesis of IBD. Extensive heterogeneity is observed in terms of presentation, extraintestinal manifestations, and location of CD, while behaviour and response to treatment are heterogeneous in both CD and UC⁶. Furthermore, it is now undisputed that enteric bacterial flora plays a key role in the pathogenesis of IBD, both in UC and CD. Nonetheless, the exact mechanism by which the intestinal mucosa loses tolerance for its bacterial neighbours remains elusive.

According to the traditional view on the pathogenesis of IBD, intestinal inflammation is mediated by the acquired immune system. The chronic inflammation could result from an overly aggressive activity of effector lymphocytes and proinflammatory cytokines, which may overcome the control mechanisms. Alternatively, IBD may result from a primary failure of regulatory lymphocytes and cytokines, such as interleukin 10 (IL-10) and transforming growth factor beta (TGF- β), to control inflammation and

effector pathways. In addition, an important mechanism in CD is the resistance of T cells to apoptosis after activation. Recently, this hypothesis has been challenged, in part, based on the results of genetic studies. The equally important role of the host's genetic regulation of the innate immune response in the pathogenesis of CD has been brought to sharp focus by the identification of the role of the NOD2 (CARD15) gene by three independent groups in 2001. As a consequence, novel genetic determinants of susceptibility and phenotype are currently being widely investigated by genome-wide scanning and studies of positional, and possibly functional, candidate genes in identified susceptibility regions of certain chromosomes (loci)⁷.

Furthermore, currently no simple diagnostic laboratory tool exists for diagnosing IBD. Biological markers that are potentially useful in IBD include proteins of inflammation such as C-reactive protein (CRP), fecal calprotectin, and several antibodies. Nonetheless, these biomarkers have many limitations. The role of the various antibodies in the current IBD diagnostic algorithm is often questioned due to their limited sensitivity⁸. In contrast, the association of serological markers with disease behaviour and phenotype is becoming increasingly well established, while other markers (e.g. CRP, EXR, calprotectin) are mainly helpful in assessing disease activity and predicting relapse.

Q to A: what is EXR?

Finally, the sequence of immunological events underlying the inflammatory reaction in IBD is extremely complex and involves both the innate and antigen-driven adaptive immune systems. Evidence indicates that dysregulation of mucosal immunity in the gut of IBD patients leads to altered production of inflammatory cytokines (e.g. tumour necrosis factor alpha (TNF- α), IL-1, -2, -4, -5, -10, -12, and more recently IL-17 and IL-23) and trafficking of effector leukocytes into the bowel (see Figure 1). The latter mechanism culminates in uncontrolled intestinal inflammation. Novel biological therapies are directed against several key players in this cascade. Major targets for such treatment include inflammatory cytokines and their receptors, as well as adhesion molecules. Blockade of T-cell proliferation and activation, and inhibition of T-cell-derived cytokines, has been most extensively targeted by clinical trials in humans in both CD and UC. Inhibition of adhesion molecules and the use of selected growth factors also seem to have a therapeutic potential. In addition, restoration of regulatory T-cell and dendritic-cell functions is still pending further investigation in clinical trials. Thus, recent advances in understanding the immunological events of IBD have led to the discovery of novel targets of biological therapy directed against several key participants involved in the inflammatory cascade.

Since a detailed presentation of the mechanisms involved would go beyond the scope of this review, and the clinical importance in everyday practice is discussed above, we will focus on emerging data concerning the genetics, genotype-phenotype associations as well as data available on biomarkers for the diagnosis and prediction of disease course in IBD. By assessing these markers we strive for an objective measurement of disease activity as symptoms may often be subjective; and secondly, to possibly avoid invasive (endoscopic) procedures that are often a burden on the patient. In this review we attempt to answer the following questions:

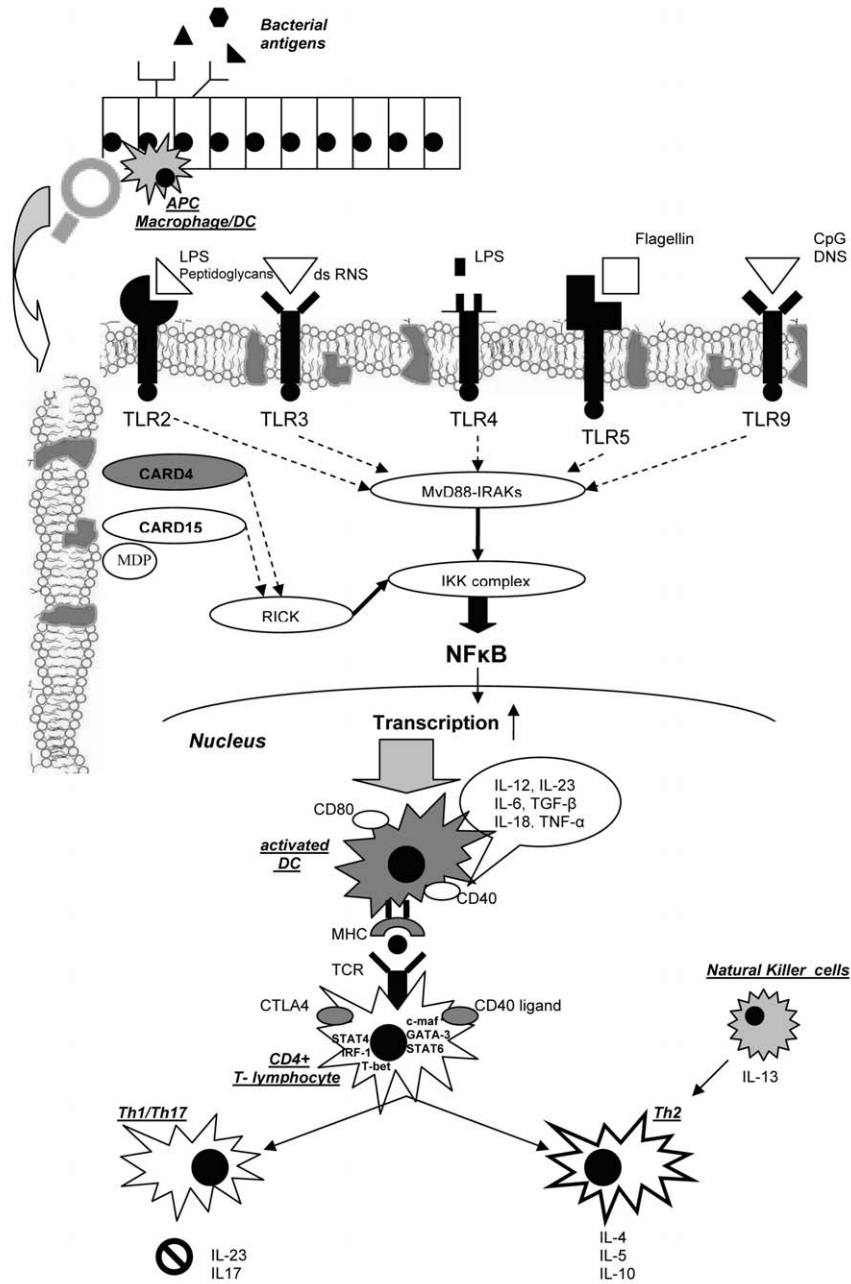


Figure 1 Pathogenesis of IBD (modified after ??)

Q to A: Which reference is this?

PATHOGENESIS AND CLINICAL PRACTICE IN GASTROENTEROLOGY

1. Is genetic investigation and detection of biomarkers helpful in the diagnosis of symptomatic IBD patients?
2. Is genetic testing indicated in clinically unaffected family members to identify patients at higher risk who might require 'preventive measures'?
3. Do genetic tests and biomarkers help in further classifying indeterminate colitis patients?
4. Can genetic tests and biomarkers help in predicting disease phenotype and progression (e.g. location, behaviour in CD, extraintestinal manifestations, need for surgery)?
5. Do genetic tests and biomarkers help in predicting relapse or response and side-effects of medical therapy, that is, do they help in 'individualizing' the treatment?

GENETICS

To this date, a total of nine loci have been found to be associated with IBD, having specific linkage requirements, and were subsequently labeled as IBD1-9⁵ (see Table 1). Furthermore, it is clear that some loci correlate with either UC or CD, while others are involved in the pathogenesis of both IBD forms.

Table 1 Locations of nine major loci showing linkage to inflammatory bowel disease (modified Q to A: reference? after ??)

<i>IBD locus</i>	<i>Chromosome</i>	<i>Identified genes</i>	<i>Disease</i>
IBD1	16q13	NOD2/CARD15	CD
IBD2	12q14	Not known (VDR, NRAMp2, MMP18, β ₂ -integrin)	UC
IBD3	6p	Not known (HLA, TNF)	IBD
IBD4	14q11-12	Not known (TCR, LTB4 receptor)	CD
IBD5	5q31-33	SLC22A4/A5 (IRGM, IL-4, 6, CD14)	CD
IBD6	19p13	Not known (ICAM1, C3, TBXA2)	IBD
IBD7	1p36	Not known (TNF-R family, IL-23R)	IBD, UC
IBD8	16p12	Not known	CD
IBD9	3p26	Not known (CCR5, CCR9, hMLH1)	IBD
	10q23	DLG5	IBD
	2q37	ATG16L1	CD

Genes involved in the recognition of bacteria*NOD2/CARD15*

Gene–protein function: In 2001 the identification of NOD2 (nucleotide oligomerization domain)/CARD15 (caspase activation recruitment domain) on chromosome 16 in the IBD1 region, as a candidate gene for CD^{9,10}, has stimulated further research focusing on novel genetic determinants of susceptibility and phenotype in IBD.

The identification of NOD2/CARD15 is of great importance in understanding the pathogenesis of IBD, especially CD, as NOD2/CARD15 is an intracellular element responsible for the indirect recognition of bacterial peptidoglycan through the binding of muramyl dipeptide^{11,12}. The function of the immune system enables it to recognize a vast number of antigens that are present in our environment (and in the gut), to mount an appropriate response as well as limit the inflammatory and immune responses elicited by different pathogens. NOD2/CARD15 serves as direct evidence that dysregulation in the immune system and recognition of bacteria can lead to immune-mediated diseases through uncontrolled inflammatory processes.

The most intriguing question that remains to be answered concerns the mechanism whereby mutations in the NOD2/CARD15 gene predispose towards the chronic intestinal inflammation characteristic of CD. Gain-of-function and loss-of-function mechanisms were both reported (e.g. increased permeability, activation of NF- κ B, regulation of Paneth cells' degranulation, regulation of TLR2 stimulation by peptidoglycans). Interestingly, mice lacking NOD2 or possessing mutated NOD2 variants do not spontaneously develop CD. Together, these data suggest that NOD2 mutations, rather than having a direct causative role in disease development, create an intestinal environment favourable for IBD¹³, for instance, via decreased expression of α - and β -defensins, thus influencing the innate immunity.

Disease susceptibility and genotype–phenotype association: Three major mutations were identified within NOD2/CARD15: one frameshift (3020insC, SNP13) and two missense mutations (R702W-SNP8 and G908R-SNP12).

The presence of a NOD2/CARD15 mutation increases the risk for CD by 1.4–4.3-fold in heterozygous patients and 17.6–44.0-fold in homozygous and compound heterozygous patients. Of importance, reports exist of homozygous individuals who are disease-free¹⁴. It is estimated that any of these three common mutations, involving NOD2/CARD15, are present in a heterozygous form in 30–50% of CD patients and 7–20% of controls from North America and Europe^{15–18}. However, various geographical differences were noted: a much lower prevalence of these mutations was reported in, for example, African American, Chinese¹⁹ and Japanese²⁰ populations. The prevalence was also lower in other northern European countries²¹. New evidence from association studies in healthy populations and disease indices, however, suggests that CARD15/NOD2 rather interacts with other, unknown risk cofactors²².

The three common NOD2/CARD15 mutations were associated with ileal disease and fibrostenosing behaviour. On the contrary, in colonic and fistulous disease, they were relatively less frequent^{10,23,24}. The mutations were not associated with UC. In CD the population attributable risk for ileal disease is determined, in 40%, by NOD2/CARD15 and in 20% by HLA genes; the numbers are similar for ileocolonic disease (NOD2/CARD15: 30%, HLA: 40%), while colonic disease is thought to be associated with HLA and other, still unknown, genes²³.

NOD1/CARD4

A protein that is structurally and functionally similar to NOD2/CARD15, the NOD1/CARD4, is located on chromosome 7p and translates into an intracellular bacterial pathogen-associated molecular pattern²⁵. Of particular interest is its location in the midst of a region with strong IBD correlation, being translated.

Conflicting data are available concerning NOD1/CARD4. Zouali et al. investigated the role of protein in 63 IBD patients. Eleven exons of the NOD1/CARD4 gene were screened for polymorphisms. Indeed, several variants were identified, none of which, according to the researchers, proved any association with IBD²⁶. In contrast, a more recent publication by McGovern et al. located a complex insertion/deletion allele on NOD1, associating it with an early onset of IBD as well as extraintestinal manifestations.

Q to A: no reference for McGovern provided

Toll-like receptors and CD14

Gene–protein function: Toll-like receptors (TLR) expressed in myeloid cells play a major role both in detecting microbes (lipopolysaccharide) and in initiating the innate immune response. Accordingly, a disturbance in their function predisposes to infections with Gram-negative bacterial pathogens^{27,28}. TLR may also influence the nature of the immune response, in particular by skewing T cells towards a Th1 or Th2 profile²⁹.

Disease susceptibility and genotype–phenotype association: IBD is characterized by an altered expression pattern of TLR on the surface of intestinal epithelium, with TLR4 expression found to be up-regulated in patients with CD. In contrast, the expression of TLR2 and TLR5 is unchanged, while TLR3, which recognizes viral replication, is down-regulated³⁰. The D299G (Asp299Gly) polymorphism of the TLR4 gene is associated with LPS hyporesponsiveness³¹ and was found to also be associated with CD (OR 2.45–2.80) and UC (OR 2.05) in the study by Franchimont et al.²⁷, as well as in Greek³² and German studies³³. However, results are conflicting, as other studies failed to replicate this association^{18,34}.

A recent study by a Belgian group³⁵ investigated 35 SNP in the TLR1–10 genes. Although none of the SNP were involved in disease susceptibility to either CD or UC, a positive association between TLR1 R80T and pancolitis in UC (OR 2.8) was found. The TLR2 R753G SNP was also associated with

pancolitis (OR 4.7). Additionally, there was a negative association between TLR6 S249P and UC with proctitis only (OR 0.22). In CD a negative association was found between ileal disease involvement and TLR1 S602I (OR 0.52).

Conflicting data have emerged on the association between the bacterial receptor CD14 and IBD. Klein et al. found the 159TT genotype to be associated with CD but not with UC³⁶. In a Japanese study³⁷ the same genotype was associated with UC (OR 1.96) but not with CD, and an additional study investigating Caucasian patients failed to demonstrate any association³⁸.

Mucosal integrity and transport

SLC22A4/OCTN1, SLC22A5/OCTN2, and OCTN3

Gene–protein function: One of the novel findings in IBD genetics in 2004 was the identification of SLC22A4/OCTN1 and SLC22A5/OCTN2 genes coding for integral membrane proteins. The proteins' functions were found to be multispecific and served as bidirectional transmembrane transporters of carnitine and organic cations³⁹. Variant alleles are associated with functional changes: the SLC22A4 variant decreases carnitine, but augments organic cation transporter activity, while the SLC22A5 variant impairs heat shock protein-driven promoter transcriptional activation⁴². Another possibility is that the enhanced cation transporter activity of the OCTN1 L503F variant may provoke disease by allowing aberrant uptake of toxic substrates.

Disease susceptibility and genotype–phenotype association: The association between 5q31 and CD was first noted in genome-wide screens (the genes for Th2 cytokines – e.g. IL-3, -4, -5, -9, -13, and IRF1 map to this region). The risk haplotype is associated with only a moderate risk for CD (OR 1.4–1.5). The effect of NOD2/CARD15 was additive; however, the 5q31 haplotype was found to be an independent risk factor^{40,41}. In a German study it was also associated with UC. Nonetheless, data support significant geographical heterogeneity as no association was found between the IBD5 haplotype and clinical presentation, nor was it correlated with IBD in a Japanese study⁴¹. Based on data available in the literature, IBD5 mainly increases the overall risk for IBD, whereas NOD2/CARD15 mutations are primarily responsible for the determination of phenotype.

The SLC22A4 C1672T and the SLC22A5 promoter G207C variants increased the risk for CD by 2–2.5-fold when present as a single copy and by 4-fold in homozygous carriers^{42,43}. The elevated risk, attributed to the OCTN TC haplotype and NOD2/CARD15 mutations, was additive with an odds ratio of 7.3–10.5 in double carriers. In a more recent Japanese study of several different SLC22A4/A5 and DLG5 polymorphisms and CD, a weak, or even no, association⁴⁴ was found with very low prevalence of variant alleles, concurring with the Ashkenazi population⁴⁵, where the frequency of the allele was also low, indicating racial differences.

In contrast, in a recent study by Vermeire et al.⁴⁶, encompassing 981 patients, no association was found between any OCTN/DLG5 variants and IBD. In this study the OCTN TC risk haplotype was associated with penetrating disease (OR 1.474, 95% CI 1.028–2.114; $p = 0.035$). In a German study⁴³ the TC haplotype was associated with non-fistulizing non-fibrostenotic disease (OR 1.57), colonic disease (OR 1.31), an earlier age at the onset of disease, and reduced need for surgery (OR 1.38).

DLG5

DLG5 is a member of the MAGUK (membrane-associated guanylate kinase) gene family, which encodes cell scaffolding proteins and is also involved in the maintenance of epithelial integrity and regulation of cell growth⁴⁷. This latter role is potentially impaired by expression of the CD-associated DLG5 variants causing increased permeability and disease.

The impact on the overall risk of developing IBD is much smaller compared to NOD2/CARD15. The association between DLG5 (*Drosophila* discs large homolog 5) at chromosome 10q22–23 was first reported by Stoll et al.⁴⁸, who showed that the DLG R30Q allele was overtransmitted to individuals with IBD (transmitted/non-transmitted, 90/73; $p = 0.09$) and CD (transmitted/non-transmitted, 58/43; $p = 0.065$). In a joint Canada–UK study Daly et al.⁴⁹ could replicate the association between R30Q and IBD in a Canadian/Italian case–control cohort; however, the association was not found in another case–control cohort from the UK. Nonetheless, the risk attributed to the variant allele was estimated to be modest (in the vicinity of 1.25). An interaction between DLG5 and NOD2/CARD15 was also detected.

This interaction, however, was not replicated by the same group in English and Scottish CD patients or by recent publications from Germany^{43,50}, Belgium⁴⁶, Norway²¹) and by our group that investigated 773 Hungarian IBD patients⁵¹. In the Belgian study, overtransmission of the wild-type allele and undertransmission of the variant 113A allele was reported. The same tendency was observed in the Hungarian patients: a protective effect for the 113A variant allele carriers (OR 0.67, 95% CI 0.45–1.013; $p = 0.06$). Thus, the contribution of this gene to disease susceptibility, if any, is relatively minor. No genotype–phenotype associations were found in the studied populations^{50,51}.

CYTOKINES, MULTIDRUG RESISTANCE AND OTHER GENE POLYMORPHISMS

Although much is known about the immune mechanisms involved in the interaction between non-pathogenic, commensal bacteria and pathogenic species, and the control of local inflammatory changes, a lack of reproducibility has challenged many of the other reported genotype–phenotype associations in IBD, such as the association between interleukin polymorphisms and IBD.

The association between a low-producing allele, allele 2 of the interleukin (IL)-1RA gene and UC (OR 1.3), extensive colitis, and the need for

colectomy^{52,53} was reported in a Caucasian population. On the contrary, another study investigating 529 IBD patients failed to confirm these results⁵⁴. Results on IL-10 and IL-18 polymorphisms are also contradictory.

In addition, an association was reported between microsatellite loci of TNF- α and CD⁵⁵. Recently the TNF receptor (TNFR) SF1A was reported to be associated with CD⁵⁶ and was less prevalent in stricturing (OR 0.38) and colonic (OR 0.41) disease. Similarly, different TNF superfamily member 15 (TNFS15) haplotypes were associated with CD in Japanese patients (OR 2.15–3.23)⁵⁷.

In 2003 Brant et al. described a suggestive linkage on chromosome 7q, containing the multidrug resistance (MDR)-1 gene, in association with the appearance of UC and CD⁵⁸. This particular gene is a membrane transport protein with several documented human polymorphisms having effects on intestinal absorption and drug pharmacokinetics. In a German study the C3435T mutation, previously associated with a 50% decreased protein secretion, but not the G2677T mutation, correlated with UC (OR 1.6–2.0). The association was strongest in extensive UC (OR 2.64) and severe disease (OR 1.67), yet it showed no manifestation in CD^{59,60}. In a recent English study⁶¹ six intronic variant SNP of MDR1 (especially the intron 3 G-A SNP), showed a very significant association to UC, particularly in patients with extensive disease. However, data are conflicting, since a Spanish group reported that the C3435T CC genotype was associated with CD (OR 1.58)⁶² and no association was found in eastern European patients⁶³.

A DIFFERENT APPROACH

The most recent studies used different methodology: instead of linkage and candidate gene analyses, broad SNP associations were investigated in genome-wide scans. The new data identified additional pieces in the puzzle of IBD.

The importance of altered bacterial sensation is underlined by the finding that an independent association scan of 19 779 non-synonymous SNP in 735 German CD cases and 368 controls has identified a significant association of an SNP in the autophagy-related 16-like 1 gene *ATG16L1* on chromosome 2q37.1 with CD⁶⁴. This SNP (rs2241880, T300A), which was associated with CD in two German panels and one British panel of CD patients (OR_{heterozygous} 1.35–1.45; OR_{homozygous} 1.71–1.77), was associated with *CARD15*. The association was not observed in a German panel of UC cases or trios, suggesting that it is specific to CD. The *ATG16L1* protein is involved in the autophagocytosis pathway, which is known to mediate resistance to intracellular pathogens such as bacteria and viral particles⁶⁵. Subsequent reports^{66,67} have confirmed the association between the variant allele and CD as well as with ileal disease in one study; however, no evidence of interaction with *CARD15*, *IL-23R*, or *IBD5* was found. Recently, an English group also reported an association between two SNP in the autophagy-inducing *IRGM* gene on chromosome 5q33.1 and CD in 2930 cases and 4962 controls. Variant SNP were associated with an OR of 1.36–1.38⁶⁸.

Q to A: please provide reference

Recently, an association was reported between rare SNP of the IL-23R gene located on 1q31⁶⁹ and CD. The variant Arg381Gln allele was associated with decreased susceptibility for CD (OR 0.26). Similar results were reported in confirmatory studies (OR 0.38–0.65 and 0.63–0.78) for both CD and UC from the UK^{70,71}. Preliminary evidence suggests an epistatic interaction with the IBD5 risk haplotype. No phenotype–genotype associations were discovered.

PHARMACOGENETICS – THE ROLE OF GENETIC FACTORS IN PREDICTING THE SUCCESS OF THERAPY AND DRUG TOXICITY

Individual response to drugs is a major problem in clinical practice. Pharmacogenetic research in IBD is developing in two directions: (1) the identification and design of possible new therapeutic targets by exploiting current knowledge of susceptibility genes – an area that is rapidly developing; and (2) examination of certain genomic areas that may be valuable in elucidating variations resulting in the lack of therapeutic benefit or adverse drug reactions, leading to a more personalized/individualized therapy. The fundamental principle underlying pharmacogenetic investigations is that variations in drug response may reflect functional differences in a gene product encoded by different alleles of the same gene. Factors may influence either pharmacodynamic (relating drug–target responsiveness at fixed drug concentrations) or pharmacokinetic (determining drug and metabolite concentrations, including absorption, distribution, metabolism, and elimination) characteristics. At the present time only limited information is available, much of it regarding the genetic basis of drug metabolism and elimination.

The genetic regulation of azathioprine/6-mercaptopurine (AZA/6-MP) metabolism has been widely studied and provides perhaps the best example of the clinical application of pharmacogenetics in IBD. Thiopurine S-methyltransferase (TPMT) limits the production of 6-thioguanines (6-TGN) by converting 6-MP to 6-thioruric acid and 6-methylmercaptopurine⁷², which may lead to myelotoxicity. Approximately 5% of the white population carries one or more variant TPMT alleles, with more than 10 variant alleles reported⁷³. The functional consequences of alleles *2, *2A, *3B, and *3C, accounting together for more than 90% of mutant alleles, have been extensively characterized.

Population studies have shown that the distribution of TPMT activity is trimodal: 0.3–0.5% of the population has low to absent activity (TPMTL/TPMTL), around 10% have intermediate activity (TPMTL/TPMTH), and approximately 90% inherit normal to high enzyme activity (TPMTH/TPMTH)⁷⁴; as previously mentioned, a correlation has been observed between the TPMT genotype and enzyme activity. Carriage of two variant alleles was associated with deficient TPMT activity, whereas heterozygosity was associated with intermediate enzyme activity. These data suggest that genotyping might identify patients at high risk of haematological toxicity. Unfortunately, a number of factors currently limit the incorporation of TPMT genotyping into clinical practice. The principal limitation is that genotyping

Q to A: is this correct definition for 6-TGN?

does not predict TPMT activity in all patients. Homozygosity or compound heterozygosity for TPMT variants is a well-known contraindication to AZA at conventional doses, as it causes early and profound bone marrow suppression.

Nevertheless, it is clear that there are many other causes of myelotoxicity. This was accurately demonstrated by Colombel et al.⁷⁵, who found that only 27% of patients had documented low TPMT activity. Other confounding genetic and environmental factors include, for instance, the patient's age, renal function, AZA formulation, coadministration of mesalazine (a reversible TPMT inhibitor) and allopurinol (XO inhibitor). Thus, the determination of TPMT activity is not an exclusive test to rely on. It may be helpful only in identifying a certain group of high-risk patients but, as the negative predictive value is rather low, it is not beneficial in ruling out possible side-effects. Also, as the prevalence of double carriage of variant TPMT alleles is as low as 1/300, continuous monitoring of red blood cell counts remains mandatory in clinical practice.

The clinical usefulness of arylamine-N-acetyltransferase 1 (NAT1), glucocorticoid receptor (GCR), MDR1 gene, DLG5, TNF, Fas-ligand, NOD2/CARD15, or 5q31 in predicting response to 5-aminosalicylate, glucocorticoids or infliximab is even more controversial.

Biomarkers

Serological markers

Anti-neutrophil cytoplasmic antibody: The classical anti-neutrophil cytoplasmic antibody (ANCA) tests are used to diagnose and monitor the inflammatory activity in primary small vessel vasculitides. On the basis of an international consensus statement, ANCA testing is performed with serum samples by indirect immunofluorescence (IIF) on normal peripheral blood neutrophils. Atypical p-ANCA are most commonly seen in patients with IBD, especially UC, and some autoimmune liver diseases such as autoimmune hepatitis and primary sclerosing cholangitis.

Atypical p-ANCA is present in the sera of 40–80% of patients with UC^{76,77} and to a lesser extent in CD (5–25%)⁷⁸. The prevalence of the antibody is also high in patients with primary sclerosing cholangitis (88%)⁷⁹ and autoimmune hepatitis type I (81%)⁸⁰, but is detected in only 1–3% of healthy control subjects. Some sera with atypical ANCA reactivity are positive for antibodies to elastase, lactoferrin, cathepsin G, lysozyme or bactericidal permeability-increasing protein (BPI). However, since they are detected in only a few atypical p-ANCA-positive sera, these antigens do not appear to be the primary targets of atypical p-ANCA reactivity. The exact target antigen(s) of atypical p-ANCA has not yet been identified.

An alternative method for the identification of atypical p-ANCA reactivity developed by Targan and colleagues uses a three-step process that includes enzyme-linked immunosorbent assay (ELISA) analysis, IIF assay on methanol-fixed neutrophils, followed by another IIF testing on deoxyribonuclease (DNase)-treated neutrophils. DNase-sensitive p-ANCA (i.e. not detectable on DNase-treated neutrophils) is present in the sera of 60–80% of the patients with

UC and in approximately 10–30% of CD patients. In approximately 70% of UC sera there is ablation of the p-ANCA pattern and antigen recognition after DNase digestion of the substrate cells⁸¹. This suggests that the epitope recognized by the UC-associated atypical p-ANCA is a protein–DNA complex, or that the presence of intact DNA is necessary for maintaining the integrity of the epitope. In up to 30% of the sera there is conversion to homogeneous cytoplasmic staining, while in 3% of the sera the p-ANCA pattern was retained after DNase treatment of the substrate.

The overall specificity of atypical p-ANCA is 84–95%, with a sensitivity of 48–63%, a positive predictive value (PPV) of 69%, and a negative predictive value (NPV) of 89%⁸.

Anti-Saccharomyces cerevisiae antibodies: The other ‘conventional’ markers, anti-*Saccharomyces cerevisiae* antibodies (ASCA) are antibodies directed primarily against a 200 kDa phosphopeptidomannan cell wall component of the common baker’s or brewer’s yeast *S. cerevisiae*⁸², eliciting the formation of both IgA and IgG antibodies. Separate and polyvalent ELISA configurations are available for ASCA IgG and IgA detection. ASCA are more frequently found in CD patients (50–80%) compared to patients with UC (2–14%) and normal healthy subjects (1–7%)^{83–85}. Approximately two-thirds of CD patients with ASCA IgG are also positive for ASCA IgA, yet 0–19% of the patients have only ASCA IgA antibodies. This finding suggests that both ASCA IgG and IgA antibodies should be measured. In CD up to 90% specificity has been reported in specimens positive for both ASCA IgG and IgA antibodies, especially when the titre of both ASCA antibodies, IgG and IgA, is high⁸⁶. Sensitivity of ASCA testing ranges from 41% to 76%, with a PPV of 88% and NPV of 68%⁸⁷.

ASCA IgG and IgA levels in CD patients are highly variable^{85,86}. The prevalence of ASCA is much higher in cases of sporadic CD and in families with only CD (63%) compared to families with both CD and UC (33%). The familial trait to ASCA is obvious, but it is questionable whether this is due to the genetic background or the effect of environmental agents during childhood predisposing to the disease.

Newly discovered serological markers: Anti-OmpC antibody is directed against the outer membrane porin C transport protein of *Escherichia coli*. The detection of the IgA antibody is done with ELISA. Anti-OmpC has been reported in 30–55% of CD patients^{85,88}, but in children and young adults it was reported in only 24%⁸⁹. The prevalence of anti-OmpC was insignificant in UC patients and in healthy subjects (5–11% and 5%, respectively). Anti-OmpC may be of value to aid diagnosis of ASCA-negative CD patients. The prevalence of anti-OmpC among ASCA-negative patients is 5–15%.

A fragment of bacterial DNA (I2), a homologue of the tetR bacterial transcriptional factor family, has been identified from lamina propria mononuclear cells in active CD and shown to be associated with *Pseudomonas fluorescens*^{90,91}. IgA anti-I2 antibodies have been detected by ELISA in IBD patients with a seroprevalence of 54% in CD and 10% in UC. Anti-I2 antibodies were also found in patients with other inflammatory enteritis (19%) as well as in healthy subjects (4%)⁷⁸.

Serological expression cloning was used by Lodes et al. to identify commensal bacterial proteins in colitic mice. The dominant antigens were found to be flagellins. Intense B-cell and CD4⁺ T-cell responses were observed against one of these flagellins (anti-CBir1). Colitis was induced when the T-cell line specific for CBir1 was transferred into naive severe combined immunodeficient mice. Approximately 50% of patients with CD have IgG serum reactivity to CBir1 versus 6% of UC patients and 8% of healthy subjects. CBir1 is the first bacterial antigen to induce colitis in animal IBD models and also leads to a pathological immune response in IBD patients⁹². Among the population of CD patients positive for atypical p-ANCA, but who do not react to other known antigens, 40–44% are positive for anti-CBir1, in contrast to UC patients who are positive for atypical p-ANCA. In this latter group it has been found in only 4% of individuals. Serum responses to CBir1 may be of help in differentiating between atypical p-ANCA-positive CD and UC patients, independently of ASCA⁹³.

Anti-pancreatic antibodies (PAB) are directed against the exocrine pancreatic tissue. The exact target antigen(s), however, have not yet been identified. These antibodies are detected using IIF on human pancreas substrate. The reported prevalence of PAB is approximately 30% in CD patients compared to 2–6% of UC patients and 0–2% of control subjects⁹⁴. The relevance of PAB in the pathogenesis of CD is unclear, and whether the presence of PAB identifies a CD subgroup also remains to be determined⁹⁵.

Patients with CD express antibodies to cell wall carbohydrate epitopes present in different pathogenic bacteria and fungi. Using a glycan array (GlycoChip) and ELISA, antiglycan antibodies have been detected in CD patients, including anti-laminaribioside carbohydrate antibody (ALCA) (18–38%), anti-chitobioside carbohydrate antibody (ACCA, 21–36%), and anti-mannobioside carbohydrate antibody (AMCA, 28%)^{96,97}. Importantly, 24–44% of ASCA-negative CD patients, in one study, were found to be positive for one or more of the antiglycan antibodies. Patients with CD who were positive for at least one of ALCA, ACCA, or gASCA (very similar to conventional ASCA) could be differentiated from UC patients, with 77% sensitivity and more than 90% specificity. The PPV and NPV were 91% and 77%, respectively. When the same criteria were applied to differentiate CD from control patients, the specificity fell to 70.3%. As one might expect, combining two or more antiglycan antibodies resulted in a higher specificity and PPV in differentiating CD from UC, but with a loss of sensitivity, NPV, and efficiency.

The prevalence of different serological markers in IBD and their association with the disease phenotype is summarized in Table 2.

Q to A: please provide reference

DIAGNOSTIC VALUE OF SEROLOGICAL MARKERS IN IBD

The role of atypical p-ANCA and ASCA as diagnostic markers for IBD appears to be limited because of their moderate sensitivity and presence in other conditions. Atypical p-ANCA can also be observed in other forms of colitis; for instance collagenous or eosinophilic colitis, and in various autoimmune liver diseases such as autoimmune hepatitis (AIH) and primary

Table 2 Prevalence of different serological markers in IBD and their association with disease phenotype (modified after ref. 8)

	Crohn's disease (%)	Ulcerative colitis (%)	Healthy subjects (%)	Clinical significance
Atypical p-ANCA	2–28	45–82	1–7	Assists in differentiating between CD and UC: Atypical p-ANCA+/ASCA-: UC Atypical p-ANCA-/ASCA+: CD CD: ASCA+: ileal involvement, complicated disease course, early need for surgery Atypical p-ANCA+: left-sided colitis, good therapeutic response, uncomplicated disease course UC: Atypical p-ANCA+: severe left-sided colitis, refractory to medical therapy, early need for surgery
ASCA	41–76	5–15	5	
Anti-Omp (IgA)	24–55	5–24	5–20	Identifies ASCA- CD patients Penetrating disease Faster disease progression Early need for surgery
Anti-I2 (IgA)	54	10	4	Inflammatory enteritis (19%) Structuring form Early need for surgery

Table 2 (continued)

	<i>Crohn's disease (%)</i>	<i>Ulcerative colitis (%)</i>	<i>Healthy subjects (%)</i>	<i>Clinical significance</i>
Anti-CBir1 (IgG)	50	6	8	Flagellin (CBir1) induces colitis in animal models of IBD Leads to a pathological immune response in IBD patients Differentiation between atypical p-ANCA+ CD and UC Small bowel involvement Penetrating and stenosing form
Antiglycan antibodies (ALCA, ACCA, AMCA)	36	3–10	0–6	44% in ASCA- patients ALCA- more penetrating; ACCA-stenosing form (small differences) Complicated behaviour, surgery?
PAB (IIF)	27–39	2–6	0–2	High specificity, low sensitivity Significance? Distinct CD subgroup?

sclerosing cholangitis (PSC)^{98,99}. ASCA has been found in autoimmune hepatitis (20%) and gastrointestinal disorders such as coeliac disease¹⁰⁰.

The combination of atypical p-ANCA and ASCA, however, may be of help in patients in whom distinction between CD and UC is not obvious with the classic diagnostic tools (patient history, radiological examination, endoscopy, and biopsy). The ASCA+/atypical p-ANCA- serological pattern is mainly characteristic of CD, while the ASCA-/atypical p-ANCA+ is characteristic of UC. Several independent studies found that these combinations had sensitivities ranging from 30% to 64%, specificity of more than 90%, and a PPV from 77% to 96%^{84,85,101,102}. It must be emphasized that neither ASCA nor atypical p-ANCA negativity rules out IBD. Similarly, the presence of these antibodies does not confirm the diagnosis of IBD.

Several groups have studied whether atypical p-ANCA and ASCA are subclinical markers of familial IBD. Some studies^{103,104} showed that atypical p-ANCA occurred frequently in healthy first-degree relatives of UC patients, whereas other studies were unable to confirm this observation^{105,106}. ASCA positivity was obviously found at a higher rate in unaffected first-degree relatives of CD patients than in the general population (20–25% vs 5%)^{107,108}. Further studies are necessary to clarify whether the unaffected, ASCA-positive, family members face an increased risk of disease development.

In addition, Israeli et al.¹⁰⁹ demonstrated that the presence of ASCA and atypical p-ANCA in healthy subjects can predict for the occurrence of IBD before the emergence of overt clinical manifestations. Serum samples were systematically obtained and stored from 5% of all military recruits. ASCA were detected in 31% of CD patients before clinical diagnosis. The mean interval between ASCA detection and diagnosis was 38 months. There was no ASCA positivity in the control population. Atypical p-ANCA was present in 25% of patients with available sera before the diagnosis of UC. None of their 24 matched controls was positive.

At last, serological evaluation may be of help in patients with indeterminate colitis (IC) to increase the diagnostic accuracy. Ninety-seven patients with IC were enrolled, analysed for atypical p-ANCA and ASCA, and followed up prospectively in a multicentre study by Joossens et al. At the end of the 1-year follow-up a definitive diagnosis was reached in 31 of 97 patients (37%). In IC patients, ASCA+/atypical p-ANCA- results correlated with CD in 80%, whereas ASCA-/atypical p-ANCA+ correlated with UC in 63%. The remaining ASCA-/atypical ANCA+ patients were eventually determined to suffer from CD, but clinically showed a UC-like CD phenotype. Remarkably, during the 9.9 years of follow-up, 48.5% of the patients did not demonstrate ASCA or atypical p-ANCA. In 85% of these seronegative patients the diagnosis remained indeterminate. In contrast, 48% of the seropositive patients were declared to have CD or UC on follow-up¹¹⁰. Adding anti-OmpC and anti-I2 to the serological panel in patients with IC did not add diagnostic clarification¹¹¹.

Recently, a sophisticated, computer-aided system was developed from a sequential analysis of serological assay results using two-stage statistical classifiers including a neural network. In the validation cohort the overall accuracy of the panel for IBD diagnosis was as high as 92% (sensitivity 93%, specificity 95%)¹¹².

ASSOCIATION WITH DISEASE PHENOTYPES AND PROGRESSION

The occurrence of atypical p-ANCA in UC is associated with a characteristic clinical appearance and represents a distinct subgroup that is often characterized by specific HLA markers. These patients have a higher probability to develop a severe left-sided UC, which is more resistant to treatment than the usual case. The disease has a more aggressive pattern requiring surgery earlier in the course of the disease¹¹³. Some authors suggest that pouchitis develops more frequently after ileal pouch anastomosis¹¹⁴, whereas others were unable to confirm this observation. The presence of atypical p-ANCA identifies a subgroup of CD patients characterized by 'UC-like' colitis; the inflammation usually involves the left side of the colon and the response to therapy is generally good. The atypical p-ANCA in CD patients is associated with a later age of onset and a relatively lower incidence of complications, such as strictures and/or perforations¹¹⁵⁻¹¹⁷.

The phenotype and the disease course of CD depend heavily on the presence and extent of the serological response targeted against various microbial antigens. In patients with an ASCA+ (IgG and/or IgA)/atypical p-ANCA-phenotype, small bowel involvement (with or without colonic disease) is more typical than the pure colonic disease (68–76% vs 34–46%)^{84,85,118,119}. ASCA positivity predicts a more aggressive disease course with a higher rate of complications. ASCA have been associated with stricturing (70%) and penetrating (51%) forms of disease as opposed to the inflammatory one (14%) and a higher risk of small bowel resection^{113,116,120}. Several studies suggest that ASCA positivity is associated with an earlier onset of disease¹¹⁵. ASCA IgA positivity in children may represent a higher risk for relapses (OR 2.9, 95% CI 1.33–6.33)¹²¹.

The presence of anti-OmpC in adult CD patients is associated with increased prevalence of the penetrating form only^{87,116,117}, while in children both the penetrating and stenosing forms¹²² are more frequent. Moreover, antibody positivity may lead to a more aggressive course of disease and a higher risk for surgical interventions.

Like ASCA and anti-OmpC, anti-I2 also appears to be associated with an increased risk for complications in adult CD patients. It is an independent risk factor for the development of the stenosing form and the need for surgical interventions^{85,87,116,117}.

Recent research has shown that the anti-CBir1 antibody is associated with ileal involvement in adult CD patients independent of other serological markers, and it predisposes to the development of both stenosing and penetrating forms⁹².

Among the antiglycan antibodies in CD, ALCA is more often positive in the penetrating form (34% vs 25%) and ACCA in the stenosing one (29% vs 18%) when compared to the inflammatory type. Nonetheless, the differences are small. No correlation was found between antiglycan positivity and the need for small bowel resections.

The number of antibodies produced against microbial antigens in CD shows a positive correlation with the severity of the disease. Mow et al.¹²⁰ retrospectively analysed 303 patients and found that the simultaneous

presence of three antibodies (ASCA, anti-OmpC, and anti-I2) resulted in an increased risk of complications (stenosing form 72% vs 23%, penetrating form 58.7% vs 27.9%, and the need for surgical intervention 72% vs 23%, as compared to the seronegative group. When all three antibodies were present, the OR was 8.6 (95% CI 4.0–18.9).

In addition to qualitative correlations, quantitative correlations with serological responses are also present. Patients expressing high titres of serological markers are more likely to have a complicated course of small bowel CD. In a prospective paediatric cohort ($n = 196$), Dubinsky et al.¹²² found, as did Mow et al.¹²⁰, that the presence and magnitude of immune responses to microbial antigens (ASCA, anti-OmpC, anti-I2, and anti-CBir1) are significantly associated with more aggressive disease phenotype. The risk of developing penetrating and/or stricturing CD was increased 11-fold in those individuals with immune responses to all four microbial antigens compared to seronegative cases (95% CI 1.5–80.4). Moreover, they demonstrated that the duration required to develop a disease complication during the 18 months of follow-up period was significantly shorter in those children who had a serological response against at least one antigen. A difference exists between cohort studies performed in children and adults, indicating variations with regard to which immune response has the greatest effect on disease course in children and adults. The explanation for this variability is not yet known.

The presence and elevated titre of several antiglycan antibodies also predisposes to the development of complicated ileal CD⁹⁷. In patients positive for more than two antiglycan antibodies (ALCA, ACCA, gASCA), ileal involvement can be seen in 93% of the cases, as compared to seronegative patients (60%) (OR 9.0, 95% CI 3.3–24.5). High ALCA titres seem to correlate with small bowel involvement and the development of stenosing and penetrating disease forms. ACCA titres, on the other hand, demonstrated no such associations.

One should be aware of the fact that blatantly striking differences in serological response are demonstrated only in a minority of patients. About one in four of the patients are positive for several antibodies and have markedly, and simultaneously, elevated antibody titres. The proportion of seronegative patients, or patients positive for only one antigen with a low antibody titre, is approximately equal. The remaining 50% of all CD patients have an intermediate phenotype based on the serological assessments. The aim is to find new serological markers which will be utilized to identify certain homogeneous groups of patients in this 'grey zone' of disease progression and response to therapy.

SEROLOGICAL MARKERS IN THE FOLLOW-UP AND TREATMENT OF IBD

In patients with UC no correlation was found between the presence and titre of atypical p-ANCA and the activity of the disease. The titre of atypical p-ANCA remains unchanged even after a colectomy¹²³. Similarly, the presence of ASCA in CD patients is relatively constant during the course of the disease and seems to be independent of disease activity^{115,118}. As a consequence, neither atypical

p-ANCA nor ASCA is suitable for monitoring of the disease. The ALCA and ACCA titres also seem to be independent of disease activity, but rather depend on disease duration⁹⁷. The prevalence of ASCA, anti-I2, anti-OmpC, and the presence of multiple serological responses are more frequent when the disease persists for a long period of time^{116,124}.

Landers et al. discovered that, following anti-TNF- α treatment, the prevalence and titre of various antibodies (ASCA, atypical p-ANCA, anti-I2, and anti-OmpC) remained unchanged in the majority of patients⁸⁸. Mesalazine treatment also leaves the ASCA level unchanged in active CD patients¹²⁵. ASCA positivity remains even after steroid treatment, yet the antibody titre declines¹²⁶.

The role of the serological response in the prediction of therapeutic effectiveness is yet to be determined. A Belgian study involving 279 CD patients failed to find any correlation between the atypical p-ANCA or ASCA positivity and the rate of response of patients given anti-TNF- α treatment. The investigators observed a generally poorer responsiveness in the case of atypical p-ANCA+/ASCA- status, but the difference was insignificant¹²⁷.

Patients exhibiting serological responses directed against various microbial antigens (OmpC and I2) should expect a higher remission rate if the budesonide treatment was supplemented with ciprofloxacin and metronidazole, while in the anti-OmpC/I2 seronegative group budesonide treatment alone proved to be more effective¹²⁸. The study raises the possibility that certain antibiotics are more effective in those CD patients who present with a marked immunological response against microbial antigens. This group of patients may be the one that can be most effectively treated through manipulation of the bacterial flora.

SEROLOGICAL MARKERS AND RECEPTORS PARTICIPATING IN INNATE IMMUNITY

Genetic heterogeneity may be responsible for the differences found in the serological response. Literature references are divided regarding the possible correlation between antibody production in patients with CD and the NOD2/CARD15 status. Several studies found serological response (atypical p-ANCA, anti-I2, anti-OmpC, ASCA IgG/A) to be independent of the NOD2/CARD15 status^{117,120}. Belgian and Hungarian work groups, on the other hand, confirmed the proposed association in large ($n = 913$ and $n = 658$, respectively) cohorts of patients. When more than one NOD2/CARD15 mutation was present, the investigators found the rate of ASCA, gASCA positivity and their titres to be significantly higher. Moreover, they found a positive correlation between the number of mutations and the prevalence of ASCA, gASCA, and ALCA, which may indicate a gene dose effect^{85,97}.

Investigating the association of the serological response with the toll-like receptor (TLR)-4 gene D299G (Asp299Gly) polymorphism led to results contrasting those found with the NOD2/CARD15 assessments. If the patient had a variant TLR4 genotype the rates of ACCA and Omp positivity were less frequent compared to the wild type, which again suggests a gene dose effect.

Polymorphisms may also play a role in the development of serological responses against the Gram-negative *E. coli* membrane protein. The Belgian work group also found a correlation between anti-Omp positivity and TLR4 gene polymorphism in patients with ulcerative colitis ($n = 272$)¹²⁷. Anti-Omp positivity was significantly lower among patients with a TLR4 variant genotype and prevalence of the antibody showed an inverse correlation with the number of variants. This was, however, not confirmed by findings of the Hungarian group for either ASCA or Omp.

SERUM AND FECAL MARKERS

C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and other laboratory markers

ESR and CRP are traditional non-specific markers for inflammation. ESR is defined as the rate at which erythrocytes migrate through the plasma. Inevitably, ESR will depend on the plasma concentration and on the number and size of the erythrocytes. Conditions such as anaemia, polycythemia, and thalassaemia affect ESR. Compared with CRP, ESR will peak much less rapidly and may also take several days to decrease, even if the clinical condition of the patient or the inflammation is ameliorated. Increases in ESR with age have also been described¹²⁹.

CRP is a pentameric protein consisting of five monomers and is one of the most important acute-phase proteins in humans¹³⁰. Under normal circumstances CRP is produced by hepatocytes in low quantities (<1 mg/L). However, following an acute-phase stimulus, such as inflammation, hepatocytes rapidly increase the production of CRP and may reach peak levels of 350–400 mg/L. Generally, CRP levels of 10–40 mg/L are found in cases of mild inflammation or viral infections^{131,132}.

Although CRP is non-specifically up-regulated in most inflammatory diseases, including IBD, there is remarkable heterogeneity in the CRP response between CD and UC. Whereas CD is associated with a strong CRP response, UC has only a modest or no CRP response in both adult and paediatric IBD populations^{133,134}. This is an important fact to keep in mind when using CRP in clinical practice. There is no good explanation for this heterogeneity. Recent studies have suggested that polymorphisms in the CRP gene, located on the long arm of chromosome 1 (1q23–24), account for interindividual differences in baseline CRP production in humans. Results are, however, conflicting, and one recent study investigating CRP polymorphisms in IBD patients showed no clear association with serum CRP levels¹³⁵.

More generally used laboratory markers include white blood cell count, platelets, and albumin. The white blood cell count will increase as part of the acute-phase response. Increased leukocytosis is therefore not a specific feature of IBD and may also be seen in other inflammatory conditions and stressful events. White blood cell count is also influenced by some treatments used in IBD, such as glucocorticoids (increased) or azathioprine and 6-mercaptopurine

(decreased). Platelet count will also increase and is therefore an indication, without being a specific marker, of inflammation. Albumin is a typical example of a negative acute-phase reactant and decreased levels may be found during inflammation. However, other conditions such as malnutrition and malabsorption may also lead to low albumin levels. Other acute-phase reactants include sialic acid, α_1 -acid glycoprotein or orosomucoid, fibrinogen, lactoferrin, β_2 -microglobulin, serum amyloid A, α_2 -globulin, and α_1 -antitrypsin, but these have not been widely studied in IBD.

A number of potentially important new serum markers could be identified by using advanced technology. Recently, protein sera profiles of 30–30 CD, UC, inflammatory and healthy controls were obtained with a surface enhanced laser desorption ionization–time of flight–mass spectrometer (SELDI-TOF-MS). This technology and the use of the multiple decision trees method led to protein biomarker patterns analysis and allowed for the selection of potential individual biomarkers with good sensitivity and specificity (minimum 80%). Four biomarkers showed important diagnostic value (PF4, MRP8, FIBA, and Hpa2)¹³⁶.

Q to A: what does 30–30 mean?

Faecal calprotectin and other faecal markers

A number of neutrophil-derived proteins present in stool have been studied, including faecal lactoferrin, lysozyme, elastase, myeloperoxidase, and calprotectin^{137,138}. Calprotectin, a 36 kDa calcium- and zinc-binding protein, is probably the most promising marker for various reasons. In contrast with other neutrophil markers, calprotectin represents 60% of cytosolic proteins in granulocytes. The presence of calprotectin in faeces can therefore be seen as directly proportional to neutrophil migration to the gastrointestinal tract. Although calprotectin is a very sensitive marker for detecting inflammation in the gastrointestinal tract, it is not a specific marker and increased levels are also found in neoplasia, IBD, infections, and polyps. Faecal calprotectin is a very stable marker (stable for more than 1 week at room temperature) and is resistant to degradation, which makes it an attractive option. Early studies using faecal calprotectin in IBD have shown a good correlation with In-labelled leukocyte excretion and intestinal permeability¹³⁹. Interestingly, increased faecal calprotectin levels have been reported after the use of non-steroidal anti-inflammatory drugs as well as with increasing age.

Further, potentially interesting markers are faecal S100A12 (calgranulin C)¹⁴⁰ and lactoferrin¹⁴¹. Calgranulin C is a member of the S100 family of calcium-binding proteins specifically expressed by granulocytes. Extracellular S100A12 exhibits proinflammatory functions, including potent chemotactic activity, comparable with other chemotactic agents. In addition, bovine S100A12 is a ligand for the receptor for advanced glycation end-products (RAGE), which is found on macrophages, endothelium, and lymphocytes. Binding of S100A12 to RAGE mediates its proinflammatory properties.

Diagnostic value of biomarkers in IBD

Only a few studies have investigated the value of laboratory markers in identifying individuals at risk for IBD. An early study from Shine et al. investigated 82 adults referred with abdominal symptoms¹⁴². In all patients a clinical examination, as well as a rectal biopsy, were performed, and ESR and CRP were determined. Of these markers, CRP was increased in all patients who subsequently were diagnosed with CD ($n = 19$), in 50% of patients diagnosed with UC ($n = 22$), yet in none of the 41 patients with functional bowel symptoms. In a paediatric study by Beattie et al., children underwent extensive blood analysis (including haemoglobin, leukocyte count, platelet count, ESR, albumin, and CRP), ileocolonoscopy, and small bowel follow-through¹³³. Twenty-six children were finally diagnosed with CD, 13 with UC, eight with polyps, seven with various gastrointestinal disorders and 37 had a normal investigation. The best laboratory marker in differentiating IBD from controls was CRP. Similar to the study by Shine and colleagues, 100% of CD patients but only 60% of UC patients had increased CRP compared with none of the children with polyps or those with a normal investigation. ESR proved to be the second-best marker. Finally, a more recent, larger study in 203 individuals referred for symptoms suggestive of lower bowel disease also showed that CRP was a good marker in differentiating IBD from irritable bowel syndrome (IBS)¹⁴³. Together, these studies seem to suggest that CRP is the most sensitive marker in detecting IBD, but values range between 50% and 60% for UC and between 70% and 100% for CD, and also depend on the cut-off value used. In addition, high-sensitivity CRP assays have been studied in other inflammatory conditions, such as atherosclerotic heart disease.

Faecal calprotectin has been shown to enable the diagnosis of IBD. In this respect a cut-off value of 30 $\mu\text{g/g}$ had 100% sensitivity in discriminating active CD from IBS in the study by Tibble and colleagues¹³⁷. In a paediatric study by Fagerberg and colleagues¹⁴⁴, 36 children with symptoms and suspected inflammation of the colon were subjected to stool analysis for faecal calprotectin and an ileocolonoscopy. Twenty-two patients showed inflammation on endoscopy (of whom 20 were later diagnosed with IBD), and calprotectin levels were much higher in these patients than in children without inflammation on endoscopy. The authors concluded that faecal calprotectin is helpful in the detection of colonic inflammation in children with gastrointestinal symptoms suggestive of IBD, and that a positive test may prioritize endoscopy. Interestingly, similarly to serological markers, increased faecal calprotectin has been described in healthy first-degree relatives of patients with CD¹⁴⁵. In a recent meta-analysis by von Roon et al.¹⁴⁶ the authors concluded that calprotectin possessed a good diagnostic value in distinguishing IBD from non-IBD diagnoses; however, at a cut-off value of 100 $\mu\text{g/g}$.

DO BIOMARKERS PREDICT DISEASE ACTIVITY, COURSE, AND EFFICACY OF TREATMENT IN IBD?

IBD follows an alternating disease course and both CD and UC are characterized by periods of remission and relapses. However, disease flare-ups occur in a random way and are often unpredictable.

CRP has been shown to be a good marker for predicting disease course and outcome in a number of diseases, thus its alteration is non-specific for IBD. Its most well-known association is with cardiovascular disease and poor outcome after myocardial infarction¹⁴⁷, and a poor prognosis in multiple myeloma¹⁴⁸.

In general, patients with severe disease more often have abnormal inflammatory markers, compared with patients without or with only low-grade inflammation. This has been shown in a prospective study by Tromm and colleagues¹⁴⁹, who investigated laboratory markers, including ESR, serum albumin, α_1 -proteinase inhibitor, cholinesterase, CRP, and haematocrit, and correlated them with endoscopic activity. One of the early studies in IBD showed a good correlation between ESR and clinical activity¹⁵⁰. The correlation was, however, dependent on disease location, and ESR did not correlate as well with UC restricted to the rectum and with CD restricted to the upper small bowel. The study by Fagan et al. showed that both CRP and ESR correlated well with disease activity but the correlation was better for CRP¹⁵¹. In addition, the correlation of laboratory markers with disease activity has been shown to be much stronger for CD than for UC. However, a wide range of CRP values was observed and no clear cut-off values exist between mild to moderate (10–50 mg/L), moderate to severe (50–80 mg/L), and severe disease (>80 mg/L). Therefore, the comparison of individual CRP values with previous values in any given patient is of great clinical importance. Lastly, CRP showed acceptable correlation with endoscopic and histological activity in CD¹⁵². For UC, again, this correlation was weaker. In contrast, recent data from Australia suggested that a subgroup of patients with active ileal disease, low body mass index (BMI), and previous ileocaecal resection was characterized by low CRP¹⁵³.

In CD a number of studies have investigated a panel of laboratory markers in predicting clinical relapse. In a prospective study by Brignola et al. 41 CD patients with clinically inactive disease (CD activity index <150) were followed-up for 6 months using a panel of inflammatory markers (ESR, white blood cells, haemoglobin, albumin, α_2 -globulin, serum iron, CRP, α_1 -glycoprotein, and α_2 -antitrypsin)¹⁵⁴. A total of 17/41 patients relapsed. ESR, α_2 -globulin, and α_1 -glycoprotein proved best at distinguishing relapsers from non-relapsers. Based on these markers a prognostic index (PI) was calculated. Using this threshold all patients with a PI >0.35 relapsed over a period of 18 months, compared with 5/29 patients with a PI <0.35. Later, Boirivant et al. prospectively followed 101 outpatients with CD¹⁵⁵. Half of the patients had an elevated CRP value and this correlated well with clinical activity. Approximately one-third of CD patients presented with active disease despite normal CRP and one-third had high CRP levels but clinically inactive disease. The likelihood of relapse after 2 years was higher in the patients with an increased CRP compared with those who had normal CRP.

More recently, the GETAID group¹⁵⁶ proposed a simple biological score for predicting short-term relapse in CD. Multivariate analysis selected two markers predictive of relapse: CRP >20 mg/L and an ESR >15 mm/h. The relative risk of short-term relapse for patients with a positive score compared to those with a negative score was 8.0 (95% CI 2.8–22.9). Sensitivity of the score was 89% and specificity was 43% with a negative predictive value of 97%, suggesting that normal CRP and ESR could almost certainly rule out a relapse in the next 6 weeks.

Much less data exist on the value of laboratory markers in assessing disease course and outcome in UC. The well-known prospective study from Oxford evaluated 49 severe UC patients treated with hydrocortisone and/or cyclosporin ($n = 49$). On day 3 a daily stool frequency of >8 or 3–8 with increased CRP (>45 mg/L) predicted with 85% certainty the need for colectomy¹⁵⁷. Only one relatively small study investigated the role of hs-CRP in IBD¹⁵⁸. Ninety CD, 70 UC patients, and 160 controls were investigated. The coefficient of correlation between hs-CRP and the disease activity score was weak but similar in both UC (0.26) and CD (0.36).

More recently, faecal calprotectin was shown to predict relapse of CD. In a study by Tibble et al. calprotectin levels of 50 $\mu\text{g/g}$ or more predicted a 13-fold increased risk for relapse¹⁵⁹. In another study¹⁶⁰, 38 CD and 41 UC patients were investigated. All patients were in remission for a mean duration of 5 months. A baseline level of calprotectin of 150 $\mu\text{g/g}$ or more was predictive for a relapse in the next year. Although sensitivity was high for both CD (87%) and UC (89%), specificity was much lower in the case of CD (43%) compared to UC (82%). Similarly, in a recent study, faecal calprotectin and lactoferrin were able to differentiate organic colorectal disease and were associated with active disease at endoscopy, both in UC (78% and 75%, respectively) and in CD (87% and 82%, respectively)¹⁶¹; however, specificity was overall low. It is thus difficult to conclude from cut-off values based on these studies.

In addition to serum markers, faecal calprotectin also correlates well with endoscopic and histological activity in patients with UC and CD, and elevated calprotectin levels normalize once the inflammation has resolved¹⁶². The role of further possible markers (e.g. β_2 -microglobulin) is conflicting.

A change in CRP, following therapy, serves as a good parameter in assessing the effectiveness of the drug on the underlying inflammation. A decrease in CRP, in response to therapy, is objective evidence that the drug has a beneficial effect on intestinal inflammation, even in patients with small changes in their symptoms. On the other hand, persistently raised CRP indicates failure of the therapy to control mucosal inflammation. This was clearly demonstrated by the different response rates to anti-TNF- α antibodies in patients with CD.

A high baseline (5–10 mg/L) CRP value before the start of therapy was associated with a higher response compared to patients with lower CRP^{163,164}. These findings raise the question of whether CRP should be included in patient selection for future clinical trials, at least for selected drugs. Furthermore, high CRP (>20 mg/L) was found to be an independent predictor for relapse after azathioprine withdrawal in patients on azathioprine therapy for longer than 42 months¹⁶⁵. Lastly, calprotectin, at a cut-off value of 200 mg/L, at 3 months post-surgery, was useful in predicting endoscopic post-surgical recurrence in

asymptomatic patients with a sensitivity of 63% and a specificity of 75%¹⁶⁶. In contrast, in a recent Italian study¹⁶⁷, only lactoferrin correlated significantly with CRP and showed a reliable threshold value for systemic inflammation following ileocolonic resection.

CONCLUSIONS

In conclusion, the answer to the question of whether genetic, immunological or biomarkers are useful in the current everyday clinical practice, is: maybe. Some of the markers (e.g. CRP, ESR, p-ANCA or ASCA) are already widely or increasingly used, while accepting their limitations. Further prospective clinical studies are needed to establish the clinical role for other (e.g. genetic or additional serological) tests in IBD. In the future, specific diagnostic and prognostic panels that include various genetic, serological and other biomarkers, as well as clinical and environmental factors (e.g. smoking) could be the most likely approaches to diagnosis, prediction of disease course, and response to therapy in IBD; however, we have to note that adding genetic aspects will not replace proper clinical judgement by the physician, who relies on the patient's symptoms and clinical phenotype. Finally, recent advances in understanding the immunological events of IBD have led to the discovery of novel biological therapies directed against several key players involved in the inflammatory cascade.

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