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Scientific news, opinions and reports

Journal ImmunoDiagnostics

Autoimmune Markers in Gastrointestinal Diseases

Approximately 15% of the patients of primary care physicians have gastrointestinal problems. In these patients, it is of utmost importance to distinguish functional, non-inflammatory causes from the less frequent and more harmful autoimmune inflammatory diseases. However, this differentiation poses a diagnostic challenge, in most cases done with the help of invasive and expensive biopsies. Today, serological and fecal tests are available which might help to reduce the number of biopsies. In this ImmunoDiagnostics Journal, the usefulness of non-invasive test methods are discussed, such as antibodies against deamidated gliadin peptides in celiac disease, fecal calprotectin in inflammatory bowel diseases and antibodies against *Saccharomyces cerevisiae* (ASCA) in Crohn's disease.

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Autoimmunity and the gut

General practitioners are confronted with it almost daily: chronic stomach pain. It is of utmost importance to be sure that there is not an autoimmune disease underlying. Usually, a biopsy helps in the diagnosis but there are non-invasive tests which can help to avoid unnecessary biopsies.
The most frequent autoimmune gastrointestinal disease is celiac disease, appearing in about 1% of the population. Studies of the last years revealed that the measurement of antibodies against tissue transglutaminase (tTG) can be enough for a definite diagnosis of celiac disease, avoiding invasive and expensive biopsies in many cases. A second seromarker for celiac disease, antibodies against deamidated gliadin peptides, are still under discussion. They were not included in the new classification criteria but in special situations they may have a diagnostic value which is described on page 3.
Another group of autoimmune gastrointestinal diseases is inflammatory bowel diseases (IBD), mainly represented by Crohn's disease and ulcerative colitis. The level of fecal calprotectin correlates directly to the degree of inflammation in the intestines. As such, it is specifically elevated in IBD. A negative calprotectin result in a patient without alarm symptoms is reason enough to avoid endoscopy while a positive result can prioritize intestinal biopsy. Therefore, using fecal calprotectin in the diagnostic algorithm for IBD may decrease costs for the health care system significantly. Please read more about fecal calprotectin on page 6 and on the influence on health care costs on page 8.
Last but not least, Xavier Bossuyt poses on page 13 the question if there are markers to distinguish between Crohn's disease and ulcerative colitis. Antibodies against *Saccharomyces cerevisiae* (ASCA), particularly in combination with atypical perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) can provide a substantial information for this diagnostic differentation.
With our new test EliA ASCA we provide the first fully automated ASCA test, running on the same instruments as EliA Calprotectin and our well-established tests for celiac disease, EliA Celikey and EliA Gliadin^{DP}.

Enjoy reading,

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Usefulness of measuring antibodies against deamidated gliadin peptides

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Coeliac disease is triggered by the consumption of gluten-containing cereal products derived from wheat, rye and barley to which patients mount a T-lymphocyte and antibody response in both IgA and IgG classes. This immune reaction is focused on the prolamine fraction of these complex proteins, mostly on gliadins (wheat), secalins (rye) and hordeins (barley). Coeliac disease, however, is best described as an autoimmune disease against the self protein tissue (type-2) transglutaminase (TG2) with disease-specific autoantibodies targeting TG2 in the patient's tissues. The immune reaction to TG2 is governed by mucosal immune processes and typically leads to the predominance of IgA class anti-TG2 production against a narrow three-dimensional epitope surface on the protein (1). This peculiar anti-TG2 reaction is dependent on the presence of gluten in the diet and stops after the elimination of gluten from the diet. As both anti-TG2 and anti-gliadin antibodies are activity markers, their detection is valuable for the disease recognition and therapy monitoring.

Problems with conventional gliadin antibody measurements

Gliadin antibody tests have been in clinical use since the early 1960's. Initially, crude gliadin fractions were used, but as gliadin is largely insoluble in water, in the early tests gliadin was added to frozen sections of monkey oesophagus where it bound around the epithelial cells in a pemphigus-like pattern (2). Then patient serum samples were incubated with this gliadin-pretreated substrate and bound antibodies were detected with anti-IgA or anti-IgG fluorescent conjugates. This test worked reasonably well, because the added gliadin bound to keratinocyte transglutaminase (TG1) expressed in this location. Later studies showed that gliadins can be used by transglutaminases as substrates, and this intimate binding was held responsible for the generation of TG2 autoantibodies via a hapten-carrier mechanism. Although in later decades gliadin antibody tests were widely used in clinical laboratories, until the discovery of anti-TG2 antibodies in 1997, serology was not a first-line test. Instead coeliac disease diagnostics was entirely based on the histology evaluation of a small bowel biopsy sample requiring the demonstration of villous atrophy. There were a number of technical difficulties with the testing (ethanol solubility and complex natural antigens, high background with anti-IgG conjugates necessitating other enzyme linking then the usual peroxidases) which could be overcome only in a few kits, but most importantly, gliadin antibodies were positive also in some healthy people or in other conditions than coeliac disease, leading to low clinical specificity. Further, there was an unfavorable sensitivity/specificity ratio and if more fractionated or synthetic peptide sets were used, this ratio became even worse. While young coeliac children with malabsorption were often gliadin antibody positive (3), this was not the case with adults and patients with mild enteral symptoms, extraintestinal manifestations or in population screenings. In other words, a test where you can have the disease with a negative test result but you can be healthy with a positive result was clinically not very useful, and in our practice anti-gliadin tests were dispensable.

A step forward with deamidated gliadin peptide antigens

Gliadin peptides are presented via HLA-DQ2 or DQ8 molecules to T-lymphocytes. In the last decade, the rules of this presentation were clarified and it looks that certain negative charges are important for the docking into the DQ2 molecule, but not for DQ8. Further, a typical arrangement of prolines and cyclic amino acids will be seen by the T cells (Fig.1).

The negative charges can be generated by TG2 on glutamines in QXP motifs by deamidation.
A highly prevalent consensus motif in these immunogenic peptides is the PQQPFP (or PQQPYP) core sequence and it was found that its deamidated variants PEQPFP, QPEQPFP and QPEQPF are also good antigens for patient antibodies (5,6), although T cell and B cell epitopes are usually not the same in many proteins. As not all gliadin peptides are deamidated or presented via DQ2, adding further peptide sequences, such as PEQLPQFEE or QEQPFP increased the percentage of reactive coeliac disease serum samples. Based on these results it became possible to use defined synthetic peptide combinations for diagnostic purposes, which is a big

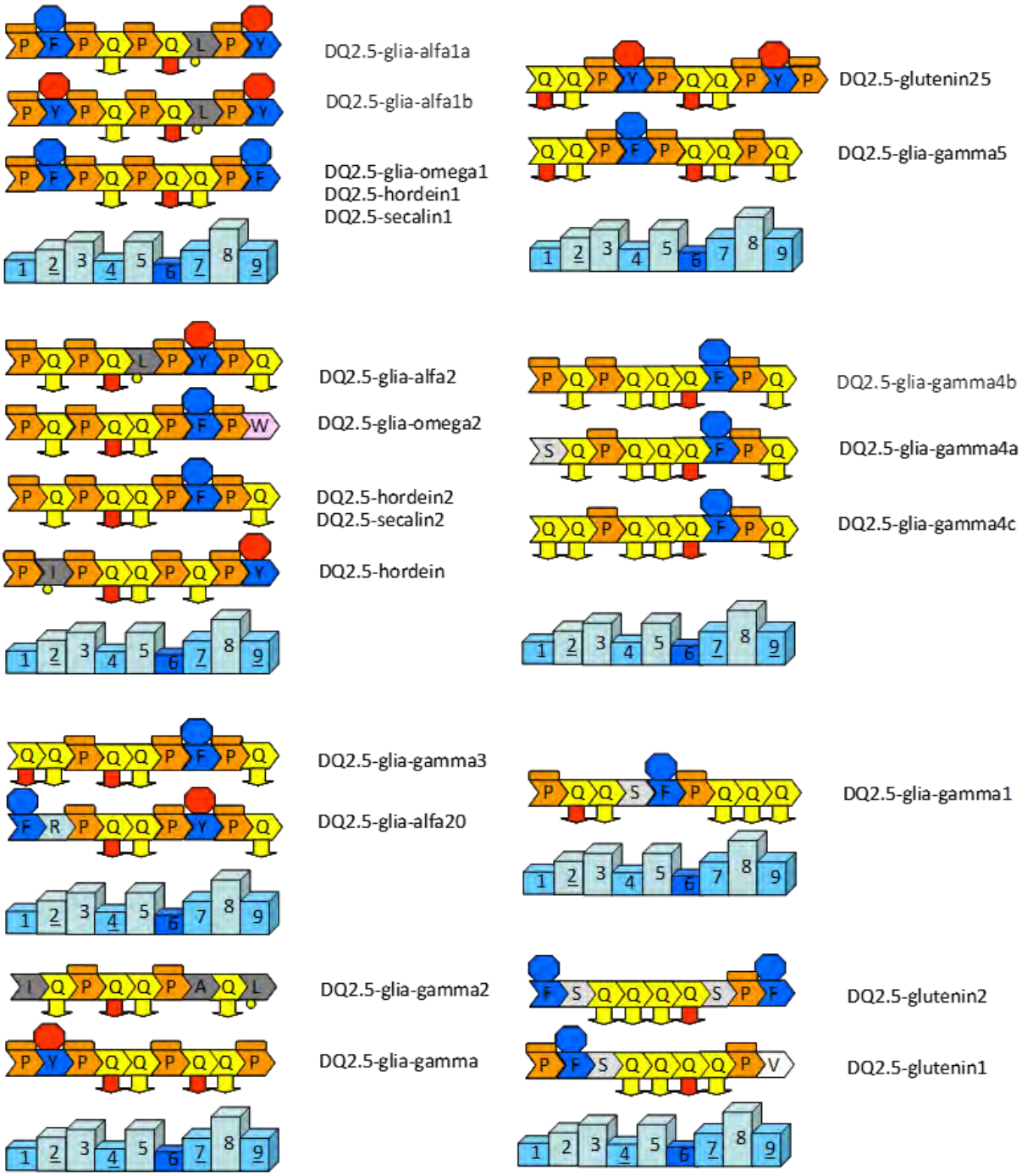

Fig.1. Gliadin peptides presented via DQ2 molecules (shown in blue at the bottom of panels). Amino acids are shown with the 1-letter code (P=proline, Q=glutamine, F=phenylalanine, Y=tyrosine, etc). Glutamines suitable for deamidation are marked with red arrows showing its docking into positions 4 and 6 of DQ2 that require acidic residue. Adapted from Camarca et al, ref.4.

advantage compared to crude gliadins. However, it must be noted that the initial studies were done with peptides synthetised on membranes and not on clinically usable antibody measuring platforms or ELISA where short peptides are not sufficiently accessible for antibodies. Therefore, clinical assays often use longer or repetitive peptide combinations, homologous protein fragments (e.g. GAF3X) or other specific proprietary binding modalitites to the assay surface, like in the deamidated gliadin antibody (DGP) assay from Thermo Fisher Scientific (EliA™ Gliadin^{DP}). Anti-DGP assays have higher sensitivity and specificity for biopsy-proven coeliac disease than traditional gliadin antibody tests and work with nearly similar efficiency as tests measuring anti-TG2 antibodies. Interestingly, in contrast to anti-TG2 antibodies where IgA class antibodies are more reliable and more sensitive, IgG class DGP seems to be more sensitive than IgA DGP assays. This fact is in line with molecular biology results that TG2 specific antibody clones were only detected in the small intestine

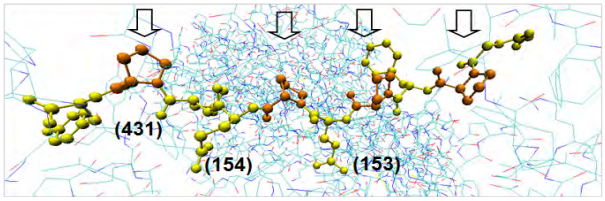

Fig.2. Crystal structure of deamidated gliadin peptide LQPFPQPELPY docked into the HLA-DQ molecule (pdb:1S9V). Numbers in brackets indicate the amino acids in TG2 taking part in the formation of the coeliac epitope with three-dimensional homology with the underlined residues in the gliadin peptide. Arrrows indicate the four proline rings (orange) seen by the T lymphocyte.

while gliadin-specific clones could be isolated also from peripheral blood indicating that the DGP antibody response is evolving on a systemic level while the antibodies against TG2 are locally produced in the gut.

Is deamidated gliadin antibody positivity related to transglutaminase antibody positivity?

In the majority of coeliac patients, antibody levels against TG2 and DGP are going hand in hand up or down during the course of disease activity, gluten-free diet and dietary lapses. Further, we observed that some non-coeliac monoclonal anti-TG2 antibodies from Thermo Fisher Scientific can recognise the DGP peptides coated to ELISA surface, and this reaction to DGP could be competed out by recombinant TG2 (7). Also when DGP-specific antibodies were affinity purified from the serum of coeliac disease patients using deamidated gliadin peptides, these purified DGP antibodies were able to recognise recombinant TG2. These experimental results indicate a certain three dimensional homology of DGP with TG2, while there is no identity with the amino acid sequence of the protein. However, the main amino acids Glu153 and Glu154 central for the coeliac epitope in TG2 (1), are spatially similarly arranged as DGP peptide glutamine and glutamic acid residues. This similarity can be important in the triggering of TG2-specific antibody response and could reveal molecular mimicry.

Deamidated gliadin peptide antibody tests in patients negative for transglutaminase antibodies

DGP antibody testing can offer clinical advantage in a number of clinical situations when the conventional anti-TG2 antibody detection is not sufficiently sensitive. As anti-TG2 antibodies are autoantibodies prone to bind to patient's TG2 autoantigen, it can be the case that they are trapped in the tissues during long-standing disease. In fact, some adult coeliac patients and young children may be negative for serum anti-TG2 and also for the endomysial antibodies (EMA) which are TG2-specific antibodies detected by immunofluorescence. As DGP antibodies do not bind to tissue TG2 in endomysial localisations (7), it is easier to detect them from the serum of such patients. Seronegative patients are rare, but we have seen a number of well documented cases which could be recognised by gliadin or DGP tests, and only became anti-TG2 positive during new gluten exposure or dietary lapses. Further, in well controlled studies, DGP and gliadin antibodies were very sensitive also in young children with coeliac disease (3).

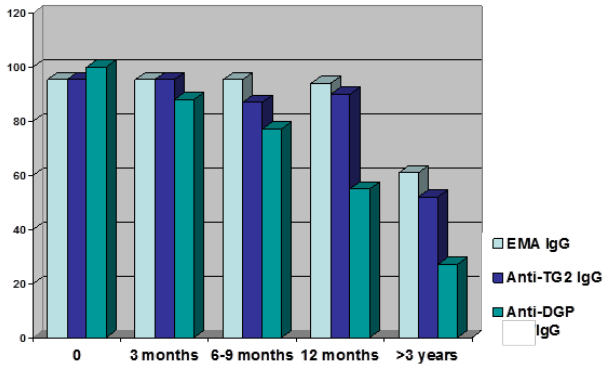

Fig.3. Percentage of IgA deficient coeliac patients remaining antibody positive after variable times on a gluten-free diet

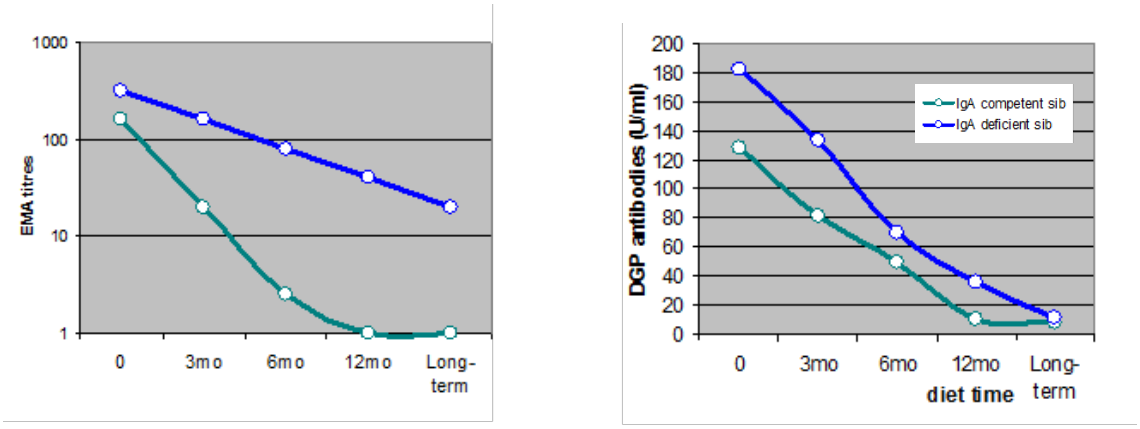

Fig.4. Decrease of IgG class endomysial (EMA) and deamidated gliadin peptide (DGP) antibodies in a coeliac sib pair judged as having similar diet compliance

Deamidated gliadin peptide antibody measurements to detect and monitor coeliac disease in selective IgA deficiency

Patients with selective IgA deficiency can be recognised by IgG class anti-TG2, IgG EMA or IgG DGP tests. As IgG anti-TG2 and EMA are often technically difficult tests, first-line use IgG DGP tests can conveniently detect these patients also in the absence of a total IgA determination. More importantly, IgG anti-TG2 and EMA are disappointing in the follow up of IgA deficient coeliac patients, as the decrease of these antibodies is very slow and sometimes not even measurable for many years.

Therefore, we investigated if recession of initially positive antibodies against DGP could be better markers of diet compliance. We followed 64 biopsy-proven coeliac disease patients and 18 coeliac sib pairs (one IgA competent and the other IgA deficient) at 3, 6, 9, 12 months after the start of the gluten-free and also on a long-term (>3 years) diet. IgG EMA and IgG TG2 were detectable in 94% and 90% of the IgA deficient coeliac patients after 1 year on diet, respectively, whereas IgG anti-DGP was detectable in 59% ($p<0.005$).

Decrease of EMA/anti-TG2 was slower and only 39% of the patients obtained negative results even after a long-term diet for more than 3 years. DGP returned to negative in 79% ($p<0.001$, Fig.3). From the 18 affected sib pairs, 15 were judged as compliant based on clinical evaluation, diet questionnaire and EMA/anti-TG2 seronegativity obtained in the IgA competent sib within 1 year. In these pairs, normalisation of IgG EMA/anti-TG2 took significantly longer time in the IgA deficient sib than in the IgA competent pair. Such a difference was not seen for DGP antibodies (Fig.4). These results indicate the superior efficiency of DGP testing to monitor disease activity in IgA deficient cases.

Deamidated gliadin peptide antibodies in the early reaction to gluten introduction in infancy

During prospective cohort studies, it was noted that antibodies against DGP or native gliadin could be first detected in infancy, before coeliac disease became manifest and before anti-TG2 antibodies appeared (8). However, when we investigated a cohort of infants, who got uniform doses of gluten at the same age in the preventCD study, almost 27% of these infants (3-56% in different countries) reacted with the transient systemic production of gliadin antibodies around 6-9 months of age (9). Some of the children had very high reactivity, even over 100 U/ml. Interestingly, all these antibodies also recognised the DGP antigens measured by EliA Gliadin^{DP} (mainly with IgG but also some with IgA antibodies) and in all investigated competitor DGP tests (n=5) as well. This DGP reaction was measurable in some cases even till the age of 1-2 years over the diagnostic cut-off. A typical time curve is shown in Fig.5.

These children were healthy, and those biopsied because of the gliadin-DGP antibody positivity or symptoms, did not have villous atrophy. Further, DGP antibody positivity at this young age did not predict the later development of coeliac disease and in a second investigated prospective cohort outside the preventCD study, it was not restricted to HLA-DQ2 or DQ8 alleles.

In order to establish if this early reactivity was specific for the deamidated moieties of the gliadin peptides in the DGP test, we also measured the serum samples of these children with homologous peptides having the same but non-deamidated sequences (Fig.6).

Using this additional test, it was found that the early antibodies recognised equally the non-deamidated and the deamidated peptides when the child was healthy, whereas the ratio of the deamidated /non-deamidated peptide increased when coeliac disease started. These results confirm that the reaction which is targeting the DGP specific moieties of the peptides is more characteristic for coeliac disease.

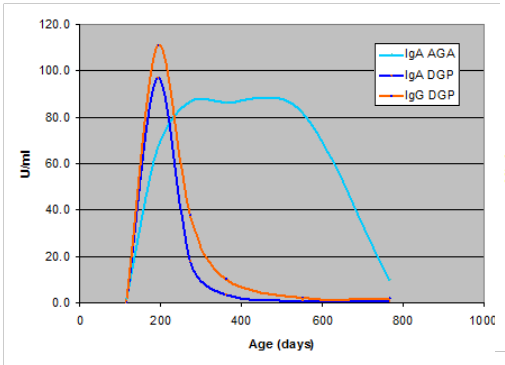

Fig.5. Serum antibody levels to gliadin peptides in a healthy infant receiving 100 mg gluten between 4-6 months of age. Long term follow up (>3 years) did not show the development of coeliac disease.

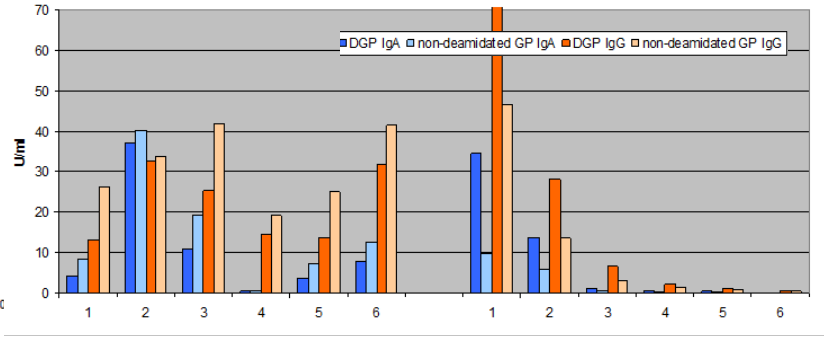

Fig.6 Reaction of children (1-6) at the age of 6 months (left panel) and at the age of 3 years (right panel) with deamidated gliadin peptides and their non-deamidated counterparts. Children 1-2 developed coeliac disease at age 3, while 3-6 remained healthy during long follow up.

Deamidated gliadin peptide antibody measurements in the new diagnostic criteria for coeliac disease

Positive anti-TG2 antibodies have high predictive value for intestinal villous atrophy and the final diagnosis of coeliac disease, especially if the serum antibody concentrations are high (for anti-TG2 >10x of the upper limit of normal). Therefore, the recently issued new diagnostic guidelines of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) allow the definitive diagnosis of coeliac disease in symptomatic childhood cases who have HLA DQ2 or DQ8 when anti-TG2 antibodies are high and this positivity was also confirmed by the more specific endomysial antibody (EMA) test. The EMA test measures the antibodies against the extracellular conformation of TG2 which are specific for coeliac disease, but the EMA test requires immunofluorescent facilities and trained observers not available in all laboratories. The new guidelines recognise the value of DGP tests to detect antibody positivity as a first line test, but it is still controversial if DGP positive results would help to establish the diagnosis of coeliac disease without a small bowel biopsy invasive test.

In laboratory studies with the combined or concomittant measurement of anti-TG2 and anti-DGP antibodies, the performance of anti-TG2 tests was not increased by the DGP results, and a smaller proportion of patients could be correctly diagnosed than with anti-TG2 alone (10). During the evidence survey ordered by ESPGHAN in 2009, both IgA and IgG DGP results had lower specificity than anti-TG2 tests (IgA DGP 86-95%, IgG DGP 86-98%), so at this stage of knowledge, it cannot be recommended to use DGP testing instead of EMA testing for a confirmatory test after a positive anti-TG2 result (11). In fact, it is not straightforward to use a test with lower specificity to confirm a positive result of an other test with higher specificity. Further, there are no high DGP antibody levels defined that would predict villous atrophy and during the early gliadin antibody reaction in healthy children even very high DGP levels were measured that were not specific for coeliac disease. In clinical cases which present with symptoms during the time interval the normal early gliadin antibody reaction can be measured, a positive DGP result may be obtained even if the symptoms are not due to coeliac disease and this is clinically misleading.

Unfortunately, even among the reports after the ESPGHAN search, none investigated this issue in biopsied patients and controls not preselected with the anti-TG2 tests, aiming at establishing a proper specificity value for the DGP tests. So the role of DGP tests remains to be seen in future prospective studies.

In conclusion, reactivity to deamidated gliadin peptides can be useful in the clinical evaluation of the patients, but also may occur as a normal reaction to food. A selective testing with a differential array of deamidated and non-deamidated peptides may reveal if this reaction is indeed DGP-specific and would indicate developing coeliac disease. This fact should be taken into account when evaluating young children, especially if they are negative for anti-TG2 antibodies. For the time being, there is no sufficient evidence that DGP antibody positivity could replace EMA in the non-invasive diagnosis of coeliac disease without small bowel biopsy.

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Faecal Calprotectin in clinical practice. Current use and the future

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Introduction

In the last 20 years there have been significant developments in the quality of endoscopic equipment and the emergence of wireless capsule enteroscopy. The whole of the gastrointestinal tract can now be visualized as a matter of routine and it is easy to obtain biopsies for diagnostic purposes. At the same time there has been a parallel increase in the availability of new biochemically based non-invasive methods for assessing gastrointestinal function. However, gastroenterologists have been somewhat reluctant to take these up for various reasons. Nevertheless endoscopy and biochemical methods provide different kinds of and complementary information with different connotations. Endoscopy with biopsy provides a diagnosis that automatically translates to treatment and may be therapeutic.

Biochemical methods on the other hand provide information on function. The potential use of such information can be summarised as:

1. Provide a diagnosis. This is the desired scope of clinical biochemistry, but is not a realistic possibility except for testing for the presence of intestinal pathogens.
2. Diagnostic screen. If of sufficient sensitivity such tests have potential as clinical screening tests that determine whether more invasive investigations are warranted. This is the main use of the tests in clinical practice.
3. Assessing responses to treatment. This is one of the main challenges in inflammatory bowel disease (IBD) where the aims of treatment are increasingly mucosal healing.
4. Providing prognostic information. Certain tests can predict an imminent clinical relapse of IBD in which case treatment can be initiated to avoid the clinical relapse.
5. For research. A number of intestinal diseases have been discovered as a consequence of acting on findings of increased intestinal permeability or inflammation in situations where no disease were thought to exist.

It is just about 20 years ago that it became feasible to measure calprotectin in faeces. The background to this discovery was the identification of this protein in serum of patients with cystic fibrosis and further characterization showed it to be largely confined to neutrophils where it accounts for a sizeable proportion of their cytosolic protein. It is resistant to bacterial degradation and as it is stable in faeces at room temperature for at least a week it had the potential to be used as a marker of intestinal inflammation. It is measured with ease in stool.

During the initial validation it was shown that total faecal excretion values over 1-4 days correlated significantly with the 4 day faecal excretion of 111Indium labelled white cells which is the gold standard for quantitating intestinal inflammation. Simplifying the procedure to the measure of calprotectin concentrations in single stool samples showed an equally good correlation with the labelled white cells.

Further validation demonstrated significant correlations between faecal calprotectin and histopathological indices of acute inflammation in patients with ulcerative colitis. Collectively this signalled the potential routine clinical use of the test and overall because there is such a significant conformity of the results from various research groups, many hospitals have taken up the test.

Of special note is that test results need to be interpreted carefully. Most centres in developed countries have an upper normal limit of the test between 50 and 60 mg/L. However Afro Caribbeans in the UK at least have normal upper limit up to 200 mg/L. Usually, in Caucasians values between 150 and 200 mg/L do not indicate a severe intestinal disease, while the vast majority of those with faecal calprotectin over 500 mg/L will have organic disease.
There follows a brief summary of the clinical and research uses for calprotectin.

Diagnosis

A point worth emphasising is that increased faecal calprotectin concentrations are inflammation- and not disease-specific. Hence it cannot be used for diagnosis of disease. However, it is by no stretch of the imagination that one can visualise a di-

agnostic biochemical panel in the future where the presence of a variety of cell specific proteins is assessed and thereby providing quantitative information of the number of cell types infiltrating the gastrointestinal mucosa and hence providing diagnostic discrimination.

Screening

The fact is that the colon is stuffed full of bacteria. Probiotic proponents talk about good and bad intestinal bacteria, but the fact is that they are all bad, ranging from relatively innocent to highly aggressive. The consequence of this is that if there is a breach in colonic integrity then these bacteria gain access to the mucosa and the body reacts with an acute inflammatory reaction, which by definition involves neutrophils. Hence inflammation is the common denominator of all significant colonic diseases. Studies from different groups show that patients with clinically active ulcerative colitis and Crohn's disease have faecal calprotectin levels that are 10-100 fold greater than healthy controls. This prompted us to assess its value in the discrimination between IBD and irritable bowel syndrome (IBS) in a patient group referred to the department of gastroenterology at King's College Hospital. 602 consecutive patients were tested for faecal calprotectin along with documentation of the ROME criteria for IBS. Overall faecal calprotectin had a sensitivity and specificity of 89% and 79%, respectively, for detecting organic intestinal disease. The sensitivity of positive Rome I criteria for IBS in this study was an impressive 85% with a specificity of 71%. However the combination of a normal faecal calprotectin and positive ROME criteria had a predictive value for IBS approaching 100%. The test can therefore be used as a screen for gastrointestinal normality and hence avoids a large number of colonoscopies if used properly.

Other noticeable diseases associated with increased faecal calprotectin is the long term NSAID ingestion leading to NSAID enteropathy, intestinal infections with Salmonella, Campylobacter and Shigella, and most interestingly is the consistent finding that the patients with colorectal cancer are abnormal by the test. The consensus from a number of studies is that the test shows a detection rate in excess of 90% for patients with colorectal cancer, irrespective of the stage of the disease (equal sensitivity in Dukes A to D). While the calprotectin test consistently outperforms faecal occult blood testing for colorectal cancer detection by a wide margin, it is not used for screening purposes as it is so non-specific. Its use for colorectal cancer screening is associated with an unacceptable increased requirement for colonoscopy (by the funders, but not the patients where curable lesions are detected).

Faecal calprotecin is normal in uncomplicated diverticular disease, but many patients with diverticulitis have raised values, especially those with segmental colitis. The test may therefore help to identify those who are likely to benefit from antimicrobial treatment, but more work is required.

The potential of faecal calprotectin in paediatric practice, where there is a reluctance to employ invasive diagnostic techniques, is clear. IBD is not particularly common in children, but the same proportion of paediatric IBD patients have abnormal results compared to the adults suggesting that it can act as a gate-keeper for more invasive procedures in this patient group.

Assessing responses to treatment

Because faecal calprotectin correlates with histopathological indices of acute inflammation in patients with ulcerative colitis (and less satisfactorily in Crohn's colitis where biopsy assessment of inflammation is highly problematic) it can be used to assess response to treatment rather than relying on clinical symptoms alone. Of particular interest are those patients who have an incomplete clinical response as a significant drop from a high to low faecal calprotectin in someone with residual symptoms should alert one to the possibility of co-existing IBS or indeed stricturing disease. However, a persistently high calprotectin in a symptomatic IBD patient that is being treated is an indication to step up the treatment.
Faecal calprotectins are increasingly used by pharmacologic companies in order to demonstrate the efficacy of new and emerging treatments for IBD.

Providing prognostic innformation

Little is known about the causes or mechanism of clinical relapse of IBD, but in perhaps 10% of cases intestinal or systemic infections, ingestion of NSAIDs, heavy alcohol binges and stress are clearly the culprit. There are a number of clinical studies that suggest that a relapse of IBD can be predicted, retrospectively, by subtle signs and symptoms of escalating increased clinical and laboratory disease activity. However the predictive value of these is far too low to be clinically useful.

The first study using calprotectin as a possible predictor for clinical relapse in patients with IBD was carried out at King's. Eighty one patients in clinical remission underwent the test and were closely followed up over the next year. Just over 50% relapsed over the next 6-12 months. The median faecal calprotectin (normal upper limit for the test kit was 10 mg/L) differed significantly between the relapse (median 123 mg/L, 95% C.I. 98-213 mg/L) and non relapse (median 32 mg/L, 95% C.I. 29-47 mg/L) groups. Receiver operator curves showed that a faecal calprotectin of 50 mg/L (a 5 fold elevation; normal less than 10 mg/L) gave the greatest sum of sensitivity + specificity. Faecal calprotectin above 50 mg/L had a sensitivity of 90% and specificity of 83% when patients with ulcerative colitis and Crohn's disease were considered

as a whole. This method outperforms all other methods for its performance in predicting relapse of disease and the results have been widely confirmed. The clinical implications are clear. Patients at minimal risk of clinical relapse of IBD may not need blank cover with 5-aminosalicylates or other drug treatment. Those at risk of relapse may benefit from immediate treatment in order to avert the relapse with all its connotations and cost.

Research

There is an interesting association between increased small intestinal permeability and the development of inflammation detected by the calprotectin test. In these circumstances further study by capsule enteroscopy has shown disease where none were otherwise expected. This is particularly so in patients with cystic fibrosis and patients undergoing various chemotherapy regimens. Many other conditions await discovery.

Conclusions

The simplicity and reliability of the faecal calprotectin method has in many ways transformed the way we think about and treat patients with IBD. Most gastroenterologists that have taken up the test appear to speak with one voice and would find the management of IBD impossible without it.
Local GP's in the area surrounding King's College Hospital have access to the calprotectin service and this use has resulted in marked reductions in referrals to the Department of Gastroenterology. Colonoscopy waiting times have been reduced drastically and a number of early colorectal cancers (Dukes A and B lesions) have been found and treated which would not have otherwise been detected at this stage (asymptomatic), but for the fact that these were invited for colonoscopy simply and only on the basis of an abnormal faecal calprotectin result. The benefits of using the test in clinical practice and research seem endless!

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Cost-Effectiveness in Diagnostic Tests: Comparison of the IBD Pre-Endoscopic Screening F-Calprotectin Test versus Serologic Markers in the United Kingdom

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The inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are characterized by chronic inflammation of the gastrointestinal tract. IBD has a low prevalence, and its incidence has significantly and consistently increased since the 1930s [2].

Irritable bowel syndrome (IBS) is a functional disorder, with a prevalence varying between 10% and 20% in the general population [3]; in the United Kingdom, it is estimated that 2.34 million people are affected by IBS [4].

IBD and IBS often present with the same symptoms, making diagnosis very difficult in primary care. Endoscopy is still considered the gold standard procedure for detecting and quantifying IBD, but, due to the low prevalence of IBD, it is usually negative in most cases of intestinal complaints. Endoscopy is expensive, and is uncomfortable and risky for the patient.

F-Calprotectin is a faecal marker of intestinal inflammation; IBD patients exhibit F-Calprotectin levels significantly higher than the general population; F-Calprotectin levels do not differ significantly in IBS patients from healthy controls [5]. There-

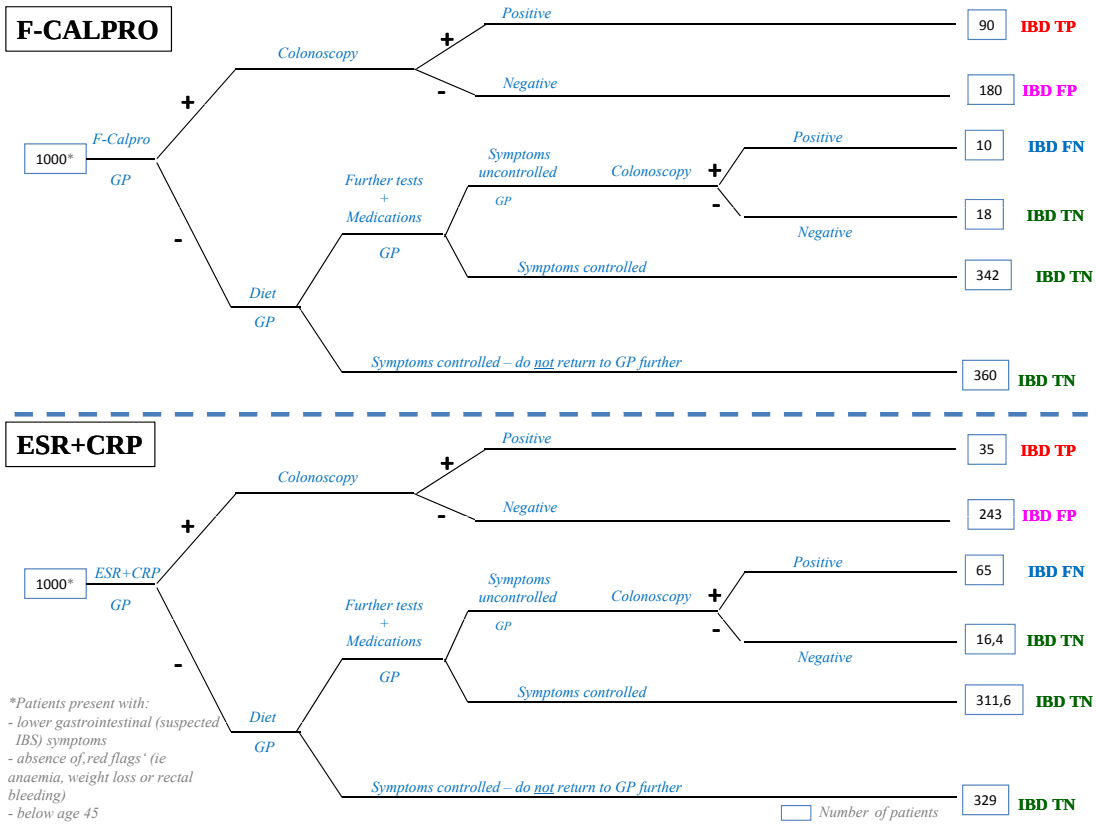

Figure 1: The NHS proposed models for F-Calprotectin (top) and ESR+CRP (bottom) [1].

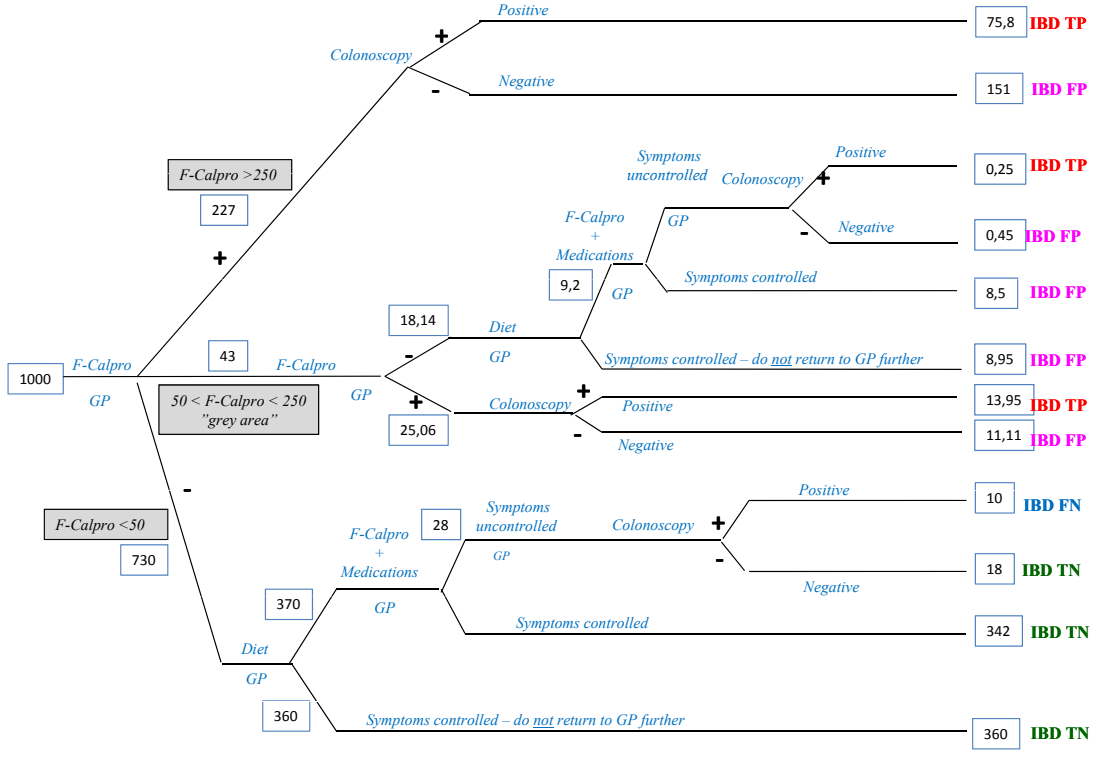

Figure 2: Refined model for F-Calprotectin.

fore, the F-Calprotectin test can be used as a pre-endoscopic technique to differentiate between IBD and IBS. Recently, NHS published an economic report [1] showing the value of F-Calprotectin in diagnosing IBD/IBS in primary care versus the standard practice, i.e. the usage of serologic markers, including the usage of CRP (C-reactive protein) and ESR (erythrocyte sedimentation rate). Results clearly showed that the F-Calprotectin strategy leads to more correct diagnoses, and to fewer unnecessary colonoscopies.

The NHS model is based on the assumption that patients with an F-Calprotectin test result between 50 µg/g and 250 µg/g are re-tested, but their second test results negative. Based on new evidence collected at Uppsala University (Sweden), the NHS model was refined allowing for a detailed description of these patients. Moreover, the sensitivity and specificity values used in the NHS report derive from a paper by Tibble and colleagues [6]; since then, the accuracy of F-Calprotectin measurement has improved significantly (Table 1). The calculations presented here are repeated using the refined model with different F-Calprotectin sensitivity and specificity input from:

a) Tibble and colleagues [6], in order to directly compare our model calculations with NHS' results [1];
b) results of a systematic meta-analysis (Table 1) including diagnostic accuracy studies [6, 8-12] and updated manufacturer's data on EliA™ Calprotectin [13].

The calculations are presented for the United Kingdom, and the costs included are the same as in [1].

Results show that the usage of F-Calprotectin is a cost-effective methodology to rule out IBD at the primary care level, and it has a higher diagnostic accuracy than CRP+ESR. The updated model shows even greater accuracy as well as cost saving:

it results in more correct IBD/IBS diagnoses at a lower price,

it reduces the number of unnecessary endoscopies because it is associated with a lower number of false positive results.

F-Calprotectin also has the potential to optimize the management of patients presenting with the described symptoms in primary care. The number of referrals to secondary care can be reduced, which results in a reduction of healthcare utilization resources, leading to a more rapid diagnosis/screening out of IBS patients.
Our results bring new evidence that F-Calprotectin is a cost-saving technique, and should be recommended for reimbursement in the United Kingdom as its cost-effectiveness meets the usually accepted standards. We are also convinced that this cost-effectiveness analysis would concretely help clinical practitioners in making decisions for the best health care of their IBD/IBS patients.

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	Sensitivity (%)	Specificity (%)	Source
F-Calprotectin	94.06 (90)	94.61 (80)	[6,8-13] ([6])
CRP+ESR	35	73	[6]
	Correctly diagnosed IBS / 1000 patients	**Correctly diagnosed IBD / 1000 patients**	**Total costs (£)/ patient**
F-Calprotectin	851 (720)	94 (90)	216.13 (274.01)
CRP+ESR	657	35	325.61
	Additionally Correctly diagnosed IBS / 1000 patients	**Additional correctly diagnosed IBD / 1000 patients**	**Incremental costs (£)/ patient**
	194 (63)	59 (55)	-109.48 (-51.6)

Table 1: Summary results of accuracy and costings of F-Calprotectin versus CRP+ESR using the refined Markov model; F-Calprotectin's sensitivity and specificity are calculated with a meta-analysis. Figures in brackets are from the F-Calprotectin sensitivity and specificity used in the NHS report.

Anti-Saccharomyces cerevisiae antibodies (ASCA)

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Introduction

Inflammatory bowel disease (IBD) denotes a heterogeneous group of disorders that involve the gastrointestinal tract, the two major entities being Crohn's disease (CD) and ulcerative colitis (UC). Although the etiology of IBD is unknown, it is thought to be an immunologically mediated disease in a genetically susceptible host. IBD most probably results from an aberrant immune response to the normal intestinal flora, leading to chronic inflammation of the gut. This idea is supported by the occurrence of antibodies directed to microbial antigens (see below) and by the identification of susceptibility genes (e.g. CARD15) that play a role in the recognition of bacterial structures.

CD patients may present with nearly any gastroenterologic symptom. Colonic disease (either alone or together with small intestine) is more frequent than disease in the terminal ileum or cecum and usually presents with chronic abdominal pain and diarrhea. Patients with CD can have perianal lesions (ulcers, fistulas). Patients with UC suffer from bloody diarrhea or rectal bleeding and tenesmus.

There are structural differences between UC and CD (1). In UC, the disease is limited to the rectum and colon. The lesions are continuous and restricted to the mucosa. There is muscular thickening, mucin depletion, and glandular damage. In CD, the disease may affect any part of the gastrointestinal tract. The lesions are discontinuous and transmural (fissure, abscess, fistula). There may be fibrosis and lymphoid ulcers. Histiocytic granulomas are characteristic for CD.

Diagnosis of IBD and differentiation between CD and UC can be made based on the combination of clinical, endoscopic, histological, and radiological criteria (2). The differential diagnosis includes irritable bowel syndrome, infectious diarrhea, carcinoma and ischemia (1). In a subset of patients with IBD (~10%), the disease cannot be classified as CD or UC and the final diagnosis is "indeterminate colitis".

Serologic markers in IBD

The most studied and best known serologic markers for IBD are atypical perinuclear antineutrophil cytoplasmic (auto) antibodies (p-ANCA) and anti-Saccharomyces cerevisiae antibodies (ASCA). P-ANCA are associated with UC, whereas ASCA are associated with CD.
The number of antibodies to autoantigens and microbial antigens associated with CD is growing. Autoantibodies targeting the exocrine pancreas are highly specific for CD, albeit with a low sensitivity (3). The target autoantigen of pancreatic antibodies in CD has been identified as GP2, the major zymogen granule membrane glycoprotein (4).
Antibodies that target microbial antigens include anti-outer membrane porin C (anti-OmpC), anti-Cbir1 flagellin and anti-I2 antibodies. New anti-glycan antibodies are directed against laminaribioside [anti-laminaribioside carbohydrate antibodies (ALCA)], chitobioside [anti-chitobioside carbohydrate antibodies (ACCA)], mannobioside [anti-mannobioside carbohydrate antibodies (AMCA)], laminarin (IgA anti-L), and chitin (IgA anti-C) (reviewed in 5). A recent meta-analysis concluded that ASCA had the highest diagnostic value among individual anti-glycan markers (6).

ASCA and atypical pANCA

Antibodies (both IgG and IgA) to the baker's and brewer's yeast Saccharomyces cerevisiae (ASCA) are found in patients with CD. The antibodies recognize carbohydrate epitopes in phosphopeptidomannan, a 200-kDa glycoprotein of the cell wall (7). ASCA are found in 40%–70% of patients with CD, in 10%–15% of patients with UC, in 4%-14% of diseased controls, and in <5% of healthy control individuals (8-12, reviewed in 13). A negative test result has a likelihood ratio that ranges between 0.2 and 0.7 (median 0.45), depending on the assay and on the study (13, 14). This indicates that a negative test result has limited clinical utility and cannot be used to exclude the diagnosis of CD, or only modestly affects the posttest probability (for tests with a likelihood ratio of 0.2). A positive test result has a likelihood ratio that varies between 3 and 5, indicating that this affects the pretest-posttest probability to a small degree. The likelihood ratio increases with

increasing antibody levels and is >20 for elevated antibody levels (observed in +/- 30% of CD patients) (14). In these cases, a positive result significantly affects the pretest-posttest probability.

Atypical p-ANCA are found mainly in UC (50%–67%), but also in CD (6%–15%) and to a lesser extent in diseased controls (<11%) (reviewed in 13). Atypical p-ANCAs are also found in autoimmune hepatitis and primary sclerosing cholangitis.

A number of studies revealed that the combination of atypical p-ANCA and ASCA may be helpful in the differential diagnosis of UC and CD in patients with IBD (8-10, 15, 16, reviewed in.13.). ASCA+/p-ANCA– is associated with CD, whereas ASCA–/p-ANCA+ is associated with UC. The combined evaluation of ANCA and ASCA had a higher specificity (>90% in most studies and >80% in all studies) to differentiate CD from UC than the separate use of either ANCA or ASCA. The increased specificity, however, was associated with decreased sensitivity. The likelihood ratio of a positive result for atypical p-ANCA and for ASCA to differentiate between UC and CD in patients with IBD was between 2 and 5 (reviewed in 13). The likelihood ratio of a negative test result for atypical p-ANCAs and ASCAs was between 0.3 and 0.7 (13). By contrast, the combined evaluation of atypical p-ANCA and ASCA had a positive likelihood ratio >5 in nearly all studies and >10 in half of the studies (reviewed in 13). This means that the combined use of atypical p-ANCA and ASCA considerably affects pretest–posttest probability in distinguishing UC from CD in patients with IBD.

Thus, the combined use of ASCA and p-ANCA could be an addition to conventional techniques (the patient's history, radiologic examination, endoscopy, and biopsy) in the differential diagnosis between CD and UC. It should be mentioned, however, that a high percentage of CD patients with pure colonic disease and UC-like colitis have been reported positive for p-ANCA (17), thereby limiting the sensitivity of ASCA+/ p-ANCA– for CD.

Serologic evaluation of ANCAs and ASCAs could be of help in patients with indeterminate colitis. In a multicenter prospective study, 97 patients with indeterminate colitis were tested for ANCAs and ASCAs (18). After a 1-year follow-up, a definite diagnosis was reached in 31 of the 97 patients. The combination ASCA+/ANCA– predicted CD in 80% of patients, whereas ASCA–/ANCA+ predicted UC in 64% of the patients. All ASCA–/ANCA+ patients who did not progress to UC developed UC-like CD. 48.5% of the patients did not have antibodies and these patients remained indeterminate after a mean duration of 9.9 years (18).

Association of ASCA with clinical phenotypes

ASCA (and the combined presence with anti-glycan antibodies) have been associated with young age at diagnosis (reviewed in 19 and 13).

ASCA have been associated with ileal disease. For example, Quinton et al. (9) found that 70% of CD patients with small bowel involvement had ASCA versus 46% of patients with pure colonic disease. Walker et al. (20) found that 68% of patients with ileum involvement had ASCAs versus 38% of CD patients with colonic disease.

ASCA have been associated with a more severe CD phenotype, such as a stricturing or penetrating disease behavior. Mow et al. (21) found an association of ASCA with fibrostenosing disease and internal perforating disease in CD patients. Amre et al. (22) found that in pediatric CD patients time to occurrence of the first complication was lower among ASCA-positive patients and among those with higher ASCA titers. Similarly, Dubinsky et al. (23) showed an association of ASCA, anti-OmpC, anti-I2 or anti-CBir1 with more rapidly progression to complicated disease than those who were seronegative. Rieder et al. (24) reported that positivity for ASCA, AMCA, ACCA, and Anti-L alone or an increasing frequency of positive serum antibodies independently predicted a faster progression toward a more severe disease course. Overall, review of the literature suggests that ASCA and an increasing number of positive antibodies is associated with more aggressive disease (19).

Several studies have demonstrated an association between the presence of ASCA and the need for abdominal surgery (reviewed in 19). ASCA status is not associated with the risk of acute or chronic pouchitis after ileal pouch-anal anastomosis (25).

A recent meta-analysis confirmed that positive ASCA are a significant risk factor for early-onset age, ileal involvement, complicated behavior, perianal disease and requirement for surgery in CD (26).

ASCA as tool for disease monitoring

The presence of ASCAs is stable over time and independent of CD activity and duration. ASCA titers most often remain stable after treatment (27-29). There is no correlation between ASCA titers and inflammatory activity or infliximab treatment (30). Hence, serial measurement of ANCA and ASCA titers in IBD is not useful for follow-up of disease activity and prediction of relapses.

ASCA in relatives of IBD patients

Several studies found an increased prevalence of ASCA in unaffected first-degree relatives of patients with CD. Sendid

et al. (31) detected ASCA in 35 of 51 (69%) patients with CD and in 13 of 66 (20%) healthy relatives versus 1 of 163 healthy controls. Seibold et al. (32) found ASCA in 25% of 193 healthy first-degree relatives. Vermeire et al. (29) found that ASCA prevalence was the same in both sporadic and familial CD. Within pure CD families, ASCA were present in 54% of CD patients with 2 family members affected vs 74.7% in CD patients with 3 or more family members affected. There was no concordance of ASCA reactivity in marital pairs.

ASCA as preclinical marker

Israeli et al. (33) demonstrated that ASCA and p-ANCA may foretell development of IBD years before the disease is clinically diagnosed. ASCA were present in 10 of 32 (31.3%) CD patients before clinical diagnosis compared with 0 of 95 (0%) controls. ASCA were positive in 54.5% of patients after diagnosis of CD. The mean interval between ASCA detection and diagnosis was 38 months. P-ANCA were present in 2 of 8 (25%) patients before the diagnosis of UC. None of the 24 matched controls were positive.

In a recent report, the blood of individuals who developed CD or UC was collected years before the onset of the disease (34). Combinations of pANCA, ASCA, anti-flagellin CBir1 and anti-OmpC were accurate in predicting incident CD and UC: 30 of 77 (39%) CD patients and 58 of 167 (35%) UC patients tested positive years before the clinical diagnosis. The predictive value of the combination of markers increased when time to diagnosis of CD or UC decreased.

Conclusion

ASCA is a marker for CD. Its role as diagnostic marker is limited, mainly due to the low sensitivity. A positive test result significantly affects post-test probability, especially when high antibody levels are found. A negative test result does not exclude disease. Current evidence suggests that serologic panels of multiple antibodies [e.g. ASCA in combination with atypical p-ANCA] might be useful in differential diagnosis of CD versus UC. ASCA titers remain stable over time and serial measurement is not useful. ASCA can be an aid in stratifying patients according to disease phenotype and risk of complications.

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- Reactivity to deamidated gliadin peptides can be useful in the clinical evaluation of the patients, but also may occur as a normal reaction to food.
- A selective testing with a differential array of deamidated and non-deamidated peptides may reveal if this reaction is indeed DGP-specific and would indicate developing coeliac disease.
- The use of calprotectin measurement has resulted in marked reductions in referrals. Colonoscopy waiting times have been reduced drastically and a number of early colorectal cancers have been found and treated which would not have otherwise been detected at this stage.
- Fecal Calprotectin is a costsaving technique, and should be recommended for reimbursement.
- The combined use of atypical p-ANCA and ASCA test results substantially affects pretest–posttest probability in distinguishing ulcerative colitis from Crohn's disease.

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