THE ROLE OF SEROLOGICAL TESTS IN REDEFINING COELIAC DISEASE

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INTRODUCTION

Coeliac disease (CD) is a permanent gluten-sensitive enteropathy characterised by small bowel mucosal atrophy. The classic malabsorptive presentation was first described by Samuel Gee in 1888 as the 'coeliac affection', but it was not until the late 1940s that Dicke, a Dutch Pediatrician, recognised that the ingestion of wheat was responsible for the harmful effect.[1] The anatomical lesion was later demonstrated in 1957 following the advent of the peroral biopsy device by Crosby and Kugler.[2] This technique has now largely been replaced by endoscopic forceps mucosal biopsy which has been shown to be safe[3] and comparable to suction biopsy in diagnosing coeliac lesions in adults;[4-6] jejunal biopsy may still be favoured in children.[7] A shift in clinical features of the disease to milder non-specific symptoms has been noted in both adults and children in the 1980s, such that the classic malabsorptive picture has now become rare.[8-10] A sensitive and specific non-invasive tool such as serological testing would be useful to select individuals to undergo small bowel mucosal biopsy for definitive diagnosis.

DIAGNOSIS OF COELIAC DISEASE

Accurate diagnosis of CD is essential as it requires a life-long commitment to a gluten-free diet (GFD). Although small bowel mucosal biopsy is the 'gold standard' on which the accuracy of serological tests are based, villous atrophy is not entirely specific to CD, especially in children where other diagnoses such as cow's milk protein intolerance and post-infective enteritis may cause confusion.[11] In 1969, the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) proposed a diagnostic protocol which required an initial characteristic biopsy while on a normal diet, histological improvement on gluten withdrawal and deterioration following gluten challenge.[11]

The protocol was simplified in 1989,[12] prompted by the availability of serological testing and evidence from the Italian Working Group for Paediatric Gastroenterology.[13] The new criteria required a characteristic initial biopsy followed by a definite, reasonably rapid, clinical remission on a strict GFD with relief of all symptoms.[12] Biopsy findings were supported by the presence of two to three positive serological tests: anti-gliadin (AGA), anti-reticulin (ARA) and/or anti-endomysium (AEM) antibodies together with their disappearance concomitantly with a clinical response.[12] The finding of occasional false-positive and negative results meant that the diagnosis could still not be established on the basis of positive serology alone.[12] Gluten challenge only remains necessary under specified circumstances.[12]

Efforts to standardise diagnosis have been confounded by the changing pattern of the disease to milder forms, the finding that not all individuals with severe mucosal abnormalities respond to a strict GFD[14] and that gluten-sensitivity is not restricted to the presence of villous atrophy.[15] Indeed, small bowel mucosal damage may progress gradually from a normal mucosal morphology to an infiltrative early lesion (raised intra-epithelial lymphocyte-IEL-count) and eventually to overt atrophy.[15] (Figures 1a and 1b) As the mucosal lesion may be patchy,[16] it has been recommended that at least three to four endoscopic biopsies should be taken to increase detection.[17,18] Although small intestinal biopsy is still the 'gold standard' for the diagnosis of CD, it is invasive, unpleasant, time-consuming and expensive.[19] Less invasive screening tests are needed to investigate patients with mild or atypical symptoms, for screening high-risk groups,[20] for timing biopsy following gluten challenge and for monitoring dietary compliance.

SCREENING OF HIGH-RISK GROUPS

Auto-immune diseases such as insulin-dependent diabetes mellitus (IDDM), Addison's disease, Graves' disease and Sjögren's disease, which share the same HLA susceptibility genes (HLA B8 and DR3) with CD, are at a higher risk of enteropathy.[21] (Table 1) Screening of high-risk groups appears to be justified by the findings that CD is associated with an increased frequency of lymphoma in the order of 40-100-fold,[22,23] and also of small bowel adenocarcinoma, oesophageal and pharyngeal squamous carcinomas.[24] A cost benefit appraisal of this approach is however lacking. Malignancy may develop in the presence of minimal mucosal pathology and a latent state.[25] Relatives of CD patients have an approximately ten-fold increased risk of malignancy compared to age-matched population controls.[25] Older coeliacs and also newly diagnosed coeliacs presenting over the age of 50 years have a one in ten chance of harbouring a lymphoma.[26]

Predictive characteristics for an enteropathy-associated T cell lymphoma include being male, a minimal or transient response to a GFD, and the absence of gliadin antibodies.[27] The diagnosis of a complicating lymphoma is often difficult and made late because of a lack of serological markers, and the presenting symptoms are similar.[27] Early diagnosis of CD in at-risk groups, who are often asymptomatic, would seem justified as adherence to a GFD for five years or more has a protective role in preventing malignancy at all sites associated with CD.[23] It is therefore important to recognise all CD patients even with minor symptoms and introduce a GFD.[23]

SELECTIVE IgA DEFICIENCY

Selective IgA deficiency (SIgAD) is the most common primary immunodeficiency, with a prevalence of 1:500 (0.2%) to 1:700 (0.14%) in the general population.[28,29] In addition to a ten-fold higher risk of CD than in the general population,[28,29] it predisposes to recurrent infection and to auto-immune diseases.[28-31] SIgAD patients have the same clinical presentation, intestinal histology and response to GFD as patients with normal serum IgA;[28,29,32] however, one study has shown a 13% increased incidence of silent forms.[33] False-negative IgA AGA and/or IgA AEM results

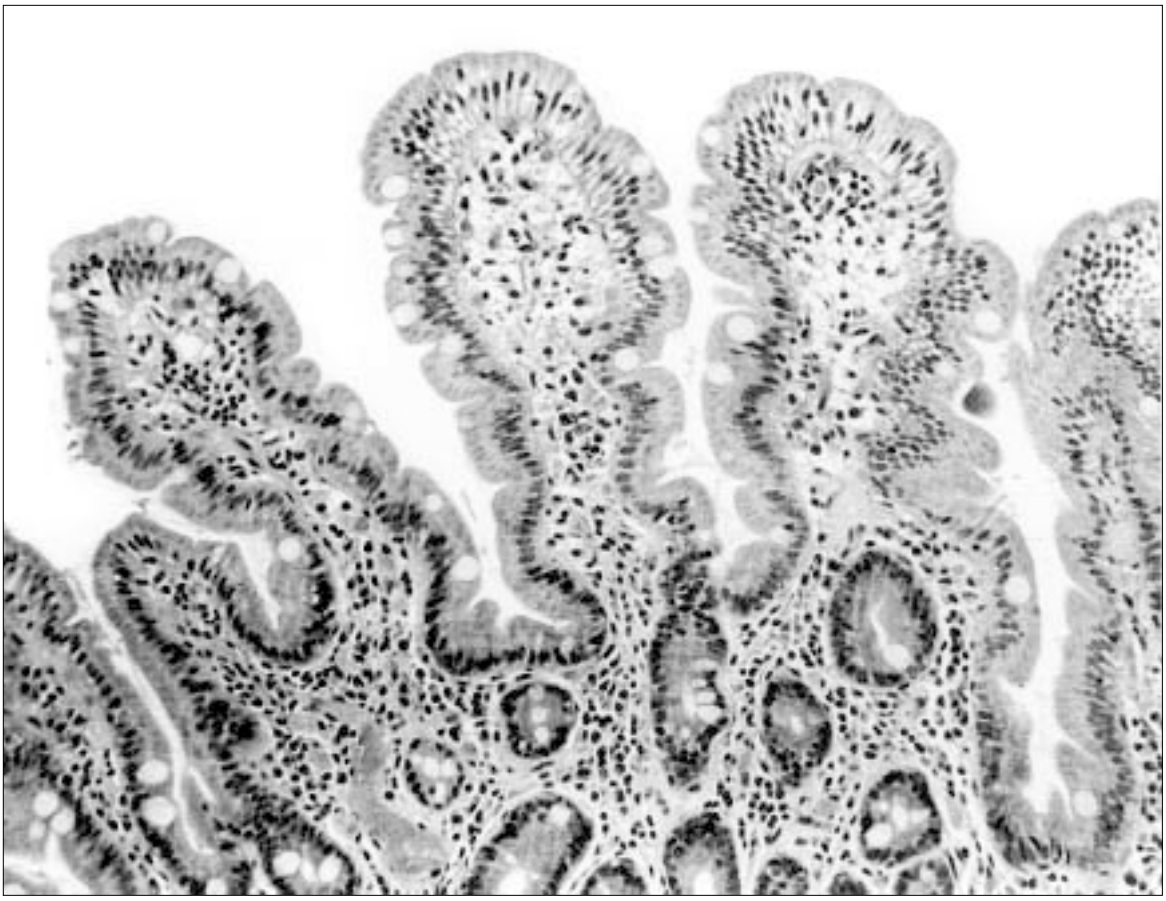

FIGURE 1A
Photomicrograph showing a normal duodenal biopsy showing preserved villous architecture (x25 magnification).

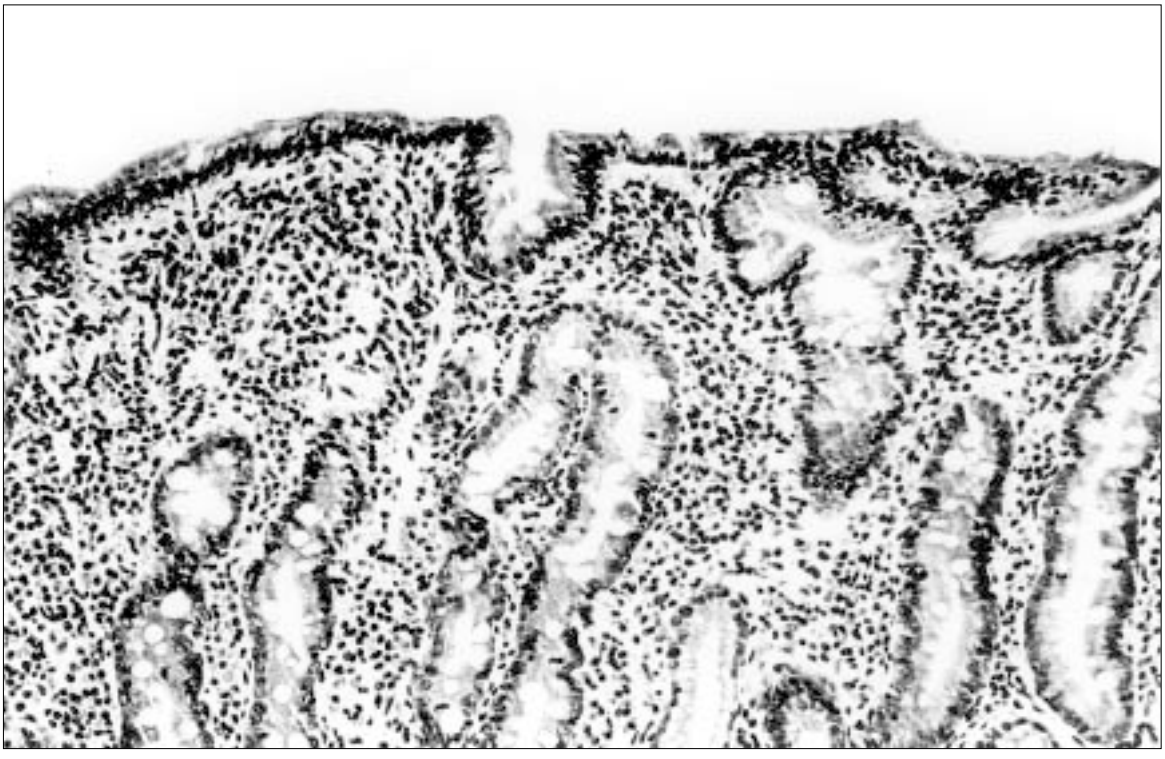

FIGURE 1B
Photomicrograph showing a duodenal biopsy from an untreated CD patient with sub-total villous atrophy, crypt hyperplasia, increased intra-epithelial lymphocytes and a dense inflammatory infiltrate in the lamina propria (x25 magnification).

TABLE 1
High risk groups to screen for CD.

High risk groups	Risk of CD	Reference
Selective IgA deficiency	10%	28
First-degree relatives of coeliac patients	10-20%	157
Dermatitis herpetiformis (DH)	30-100%	42-44
Insulin-dependent diabetes mellitus (IDDM)	5.4%	21
Sjögren's syndrome	3.3%	21
Thyroid disease	5.4%	21,181
Down's syndrome	3.9%	182
Epilepsy and cerebral calcifications	5%	183
Neurological disease of unknown cause	16%	184
Primary bilary cirrhosis	6%	185

due to SIgAD have been reported.[29,34,35] IgG AGA, ARA and/or AEM have been found to be useful in detecting these patients and in monitoring their response to a GFD;[28,33,34,36] however 6% may have a normal IgG AGA.[33] It is uncertain why SIgAD individuals are at a higher risk of CD but it is possible that genetic factors and lack of mucosal IgA reduces exclusion of dietary antigens and leads to impaired immunological tolerance.[29,33] It has been suggested that total serum IgA levels should be determined whenever screening for CD to avoid false-negative IgA serology results.[29,33,37] As some SIgAD patients may also lack IgG AGA, a small bowel biopsy should be performed whenever there is a suspicion of CD.[33]

DERMATITIS HERPETIFORMIS

Dermatitis herpetiformis (DH) is characterised by a symmetrical pruritic, blistering skin rash with granular subepidermal deposits of IgA in remote, uninvolved skin.[38] DH was first associated with CD by Marks in 1966.[39] The presence of CD is difficult to suspect as most DH patients do not have significant symptoms or laboratory evidence of malabsorption, which is thought to be due to the limited extent of the enteropathy.[40,41] The frequency of enteropathy in DH has been reported to be 30%[42,43] but this has been disputed by Brow who has shown that the enteropathy is always present if sufficient mucosal biopsies are taken.[44] DH patients with apparently normal intestinal biopsies can be induced to manifest characteristic biopsy appearances after a high gluten diet.[38,45] As the mucosal lesion may be patchy, multiple biopsies have been recommended.[16,41] The histological findings of intestinal biopsies are indistinguishable from CD[39] and both the skin and intestinal lesions respond to gluten withdrawal.[46] DH patients on a normal diet have a ten-fold increased risk of malignancy, predominantly lymphoma, compared to those on a GFD.[47]

The origin of the IgA which deposits in the skin of DH patients is unknown; however the strong association with CD has led to the hypothesis that this IgA is of gut origin.[48] AGA and ARA have both been found to have disappointingly low sensitivities of less than 50% in DH.[49-51] The overall sensitivity of AEM in DH has been shown to be between 50-65% [40,48] with specificity of 100%.[40] The sensitivities of ARA and AEM rise to 80-93%[40,48,52] and 80-100%[52,53] respectively, when patients are selected by the presence of severe villous atrophy. There is a significant correlation between the presence and concentrations of AGA,[50,51,53] ARA[48,51] and AEM[40,48,53] with the severity of the enteropathy. Antibody titres decline on a GFD and the AEM usually becomes negative within one year.[40]

ROLE OF SEROLOGY IN SCREENING FOR COELIAC DISEASE

CD is one of the most common chronic gastrointestinal conditions in European countries.[54] The prevalence of CD in European countries, based on symptomatic cases, has been estimated to be between 1:300 and 1:1,000 individuals.[55,56] The prevalence is probably higher as the classic clinical picture is now infrequent, and subtle forms are often missed.[9,54] The increased prevalence is due to greater awareness of the spectrum of presentations, screening of high-risk groups and improved diagnostic methods. It is clear that diagnosing symptomatic cases only represents the tip of the iceberg.[57,58] (Figure 2)

The predictive accuracy of serological tests depends on the disease prevalence in the population. The prevalence rate of CD in many European countries is about 0.2%.[59] At this prevalence rate, the AGA ELISA has a predictive value of only about 2%, whereas the AEM gave a positive predictive value (PPV) of close to 100% with sensitivity and specificity approaching 100%.[59] In gastroenterology out-patient clinics where the prevalence of CD has been estimated to be 47%, the PPV of IGA AGA was 71%.[59] Others agree on the superiority of the AEM test over AGA for screening in the hospital setting[20] and in population screening.[60,61] As the AEM test may be too expensive and labour-intensive for

Clinical situations where CD should be considered:

- Diarrhoea - may follow gastrointestinal infection
- Unexplained weight loss
- Anaemia - iron, folate or vitamin B_{12} deficiency
- Upper abdominal pain
- Fatigue
- Abdominal bloating
- Short stature
- Recurrent oral aphthous ulceration
- Dental enamel hypoplasia
- Osteoporosis (in the young)
- Subfertility/infertility
- Positive family history of CD
- Dermatitis herpetiformis
- Selective IgA deficiency
- Other associated disorders (See Table 1)

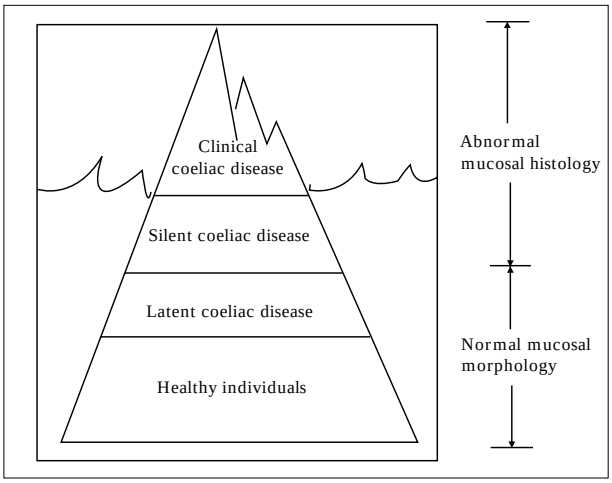

FIGURE 2
The coeliac 'iceberg' demonstrating spectrum of gluten sensitivity.

large-scale screening,[59] a two step approach has been suggested using a sensitive, cheap method such as the AGA first and then proceeding to AEM for positive sera only.[59,60] Others are opposed to a two step approach and would advise a combination of tests during the initial stage.[58] Reliance on a single antibody alone could miss up to 50% of patients with CD.[62] Currently, the GI laboratory at the Western General Hospital performs IgA AGA and IgA AEM on all samples routinely, but not IgG AGA unless there is a known history of SIgAD or the IgA AGA is very low.

Although the use of serological testing could increase the identification of CD by up to 12%[63] and increases the identification of CD in primary care,[64,65] the use of serological tests alone for screening will underestimate the prevalence of CD due to false-negative results.[20,66] The varying presentations of CD demand a high degree of clinical suspicion and alertness to make an accurate diagnosis. A proposed algorithm for diagnosing CD is shown in Figure 3.

ANTI-GLIADIN ANTIBODIES

Anti-gliadin antibodies (AGA) were first described by Berger in 1958 and were the earliest discovered serological markers associated with gluten-sensitive enteropathy.[67,68] AGA are directed against dietary gliadin which may be absorbed intact across the gut mucosa (similarly to ovalbumin) in healthy individuals.[69] Detection techniques for AGA have evolved and improved over the years.[68,70] The enzyme-linked immunosorbent assay (ELISA), first introduced by Hekkens *et al*. in 1977, has become the most commonly used technique because of its simplicity, low cost, objectivity and the possibility of batching and analysing large series of sera at a time.[68] It also allows for sub-class identification of antibodies.[71] Although gliadin is a complex mixture of proteins containing at least 40 components for a single variety of wheat, [68] the use of highly purified gliadin fractions as the coating antigen such as α–gliadin[72] or Fraction B[73] instead of crude gliadin[74,75] does not improve specificity.[76]

Serological methods used in diagnosing CD:

- Anti-gliadin antibodies (AGA)
- Anti-reticulin antibodies (ARA)
- Anti-endomysium antibodies (AEM)
- Anti-jejunal antibodies
- Anti-tissue transglutaminase antibodies
- Anti-calreticulin antibodies

AGA in coeliac patients

Untreated CD sera contain high levels of IgA, IgG and IgE AGA, but not usually IgM,[77] unless the patient is IgA deficient.[74] High concentrations of AGA are also found in intestinal secretions of CD patients and are predominantly of the IgA and IgM isotypes.[78] Serum AGA is thought to be mostly derived from the gut mucosa on the basis of its molecular size[70] and subclass distribution.[79] In practice, only IgA and IgG AGA are used diagnostically but there has been controversy over the preferred sub-class.[41]

The sensitivity of IgA AGA ranges from 46-100% and specificity from 84-100%.[37,74,75,80-88] The sensitivity of IgG AGA ranges from 55-100% and specificity from 42-97%.[37,74,80-84,86-88] (Table 2) The tests are therefore often used in combination to take advantage of the high sensitivity of IgG and the high specificity of IgA AGA.[70,71,87,89] The combined sensitivity increases to 84-100% and specificity to 80-99%.[72,75,86,87,90,91] This approach would have the added benefit of detecting CD patients with SIgAD.[71] IgA AGA may be normal in up to 16% of adult untreated CD patients,[90] but few cases have both normal IgA and IgG AGA.[71] Both IgA and IgG AGA have been shown to be very sensitive markers of untreated CD in patients under the age of two years displaying 100% sensitivity[74] compared to after the age of two when sensitivity of IgA reduces to 52-64% and IgG to 55-88%.[74,80] AGA levels increase with age in healthy individuals[41,73,92] with concentrations rising from 12% in children to 35-40% in those aged 60-70 years.[41] Raised AGA concentrations should therefore be interpreted cautiously in older age groups.[41,73]

AGA in non-coeliac individuals

AGA are not specific to CD[82] and the AGA in healthy individuals or other disease states is explained by the absorption of intact gliadin across the normal gut mucosa.[69,71] AGA are not HLA-associated[70] and do not occur more commonly in atopic individuals.[93] Follow-up studies of healthy AGA-positive individuals with normal intestinal biopsies have shown that elevated AGA titres may be transient.[94-96] Others have found that AGA-positive individuals have a higher incidence of diarrhoea, chronic fatigue and persistent/recurrent headache as well as a significantly lower serum folate, transferrin saturation, MCV and MCH, than controls.[97] It has been shown that healthy individuals with positive AGA and apparently 'normal' small bowel mucosal biopsies may have subtle abnormalities such as raised IELs.[98]

High levels of IgG AGA have been found in skin disorders including atopic eczema, pemphigus and pemphigoid,[70] and also in Sjögren's syndrome.[99] Raised levels are also seen in about 50% of patients with rheumatoid arthritis,[100] raising the possibility that these individuals are more prone to NSAID-induced damage with increased small bowel mucosal permeability to gliadin.[70] High AGA titres and IEL counts have also been observed in

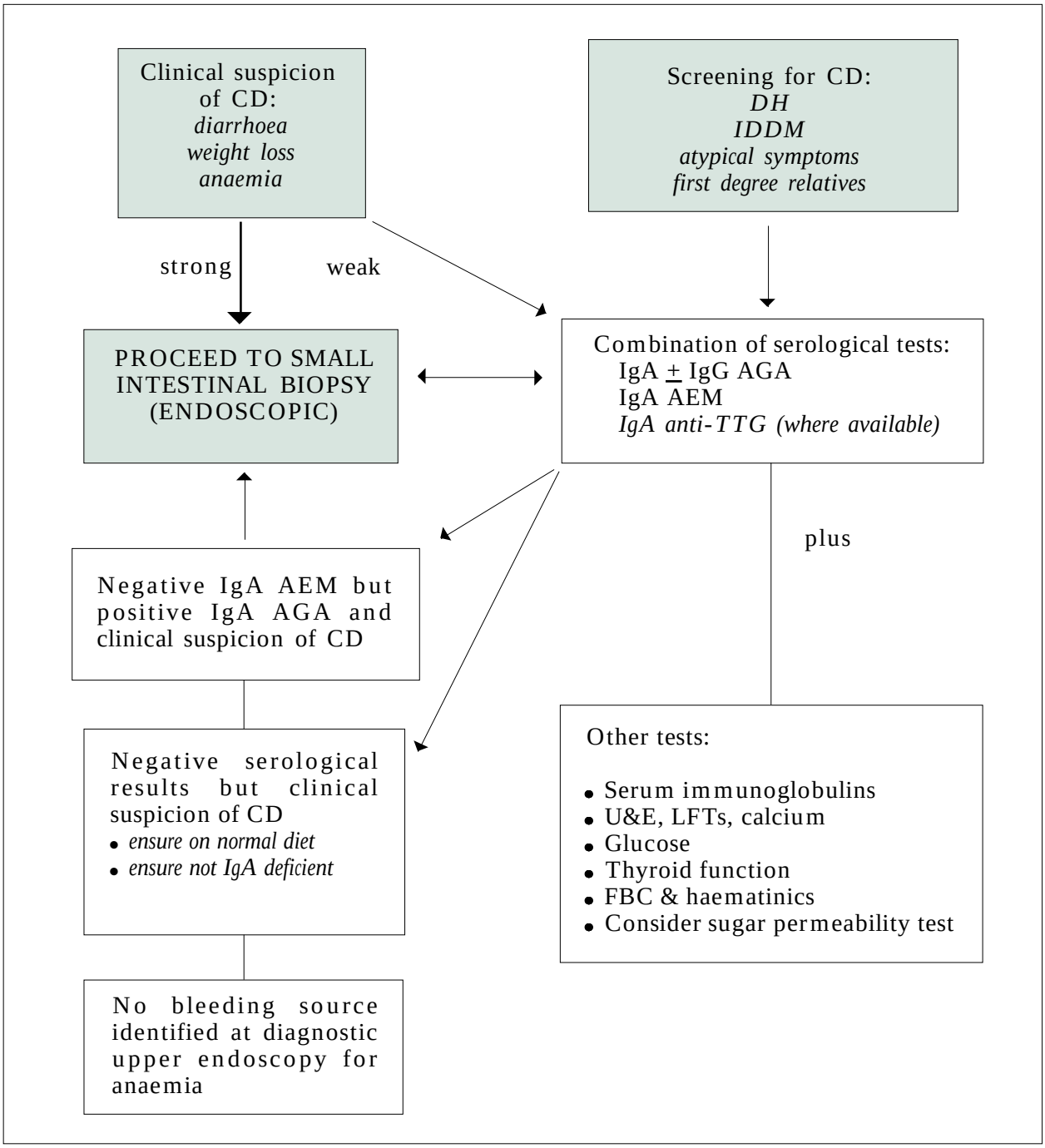

FIGURE 3
An algorithm for the diagnosis of CD.

sarcoidosis.[101] Raised IgA AGA have been found in post-infection malabsorption,[102] Crohn's disease,[74,75] cow's milk enteropathy[75] and autoimmune enteropathy,[75] in association with an abnormal small bowel mucosa. IgA AGA is thought to be a better indicator of small bowel abnormalities than IgG AGA,[74,75] but, notably, AGA is not elevated in T cell lymphoma.[27]

ANTI-RETICULIN ANTIBODIES

Anti-reticulin antibodies (ARA) were first described in adult CD and DH[103] and childhood CD[104] by Seah in 1971. ARA are detected using an immunofluorescence technique on a composite block of rat liver, kidney and stomach tissue - the same substrates used for autoantibody profile screening.[71] Although five patterns of reticulin antibody fluorescence have been described, only the original pattern designated as R1-reticulin (R1-ARA),[105] is associated with CD.[106] The different staining patterns of ARA can be difficult to distinguish. A definite positive R1-ARA result requires the pattern to be present in all three substrates.[71] If these are not all positive, the R1-ARA needs to be recorded as negative, and the serum should be tested for AGA and AEM as well.[71]

Following its initial optimism in diagnosing 85% of children with CD, the sensitivity of ARA has proved to be disappointing in several studies.[49] The sensitivity has ranged

TABLE 2
Sensitivities and specificities of serological methods in diagnosing CD.
Anti-gliadin antibodies (AGA), anti-reticulin antibodies (ARA), anti-endomysium antibodies (AEM), anti-jejunal antibodies (JAB) and anti-tissue transglutaminase antibodies (TTG) are shown.

Serological assay	Sensitivity (%)	Specificity (%)	References
IgA AGA	46-100	84-100	37,74,75,80-88
IgG AGA	55-100	42-97	37,74,80-84,86-88
IgA ARA	44-100	92-100	36,52,80,81,115,116,118,121,123
IgA JAB	93	100	132
IgA AEM	74-100	96-100	20,36,37,80,81,112,113,115,118,119,121-126
IgA TTG	84.8-98.1	91.1-98	142-145,186

from 33-97% in adults and children with untreated CD,[75,84,107,108] to 12-30% in DH,[49,107] and to 2-7% of controls.[75,107,108] (Table 2) During follow-up on a GFD, IgA ARA titres fall rapidly (usually within the first month) in parallel with the decline in AEM titre[52] and are negative in most cases after one year.[84,107,108] They re-appear on a gluten challenge.[52] The IgG ARA is less sensitive (59%) but equally specific (97%) with a PPV of 71% compared to IgA ARA PPV of 88%.[108] Volta has noted that AGA-negative samples do not show R1-ARA staining and that it is not useful to search for ARA staining in these samples.[109] Unsworth has observed that IgA R1-ARA-positive samples are always AEM-positive, but the converse is not always true.[71] ARA appears to be highly specific to CD but only in experienced hands because of difficult immunofluorescence interpretation. False-positive IgA ARA have been reported in IDDM[108] and in Crohn's disease.[49,110] The AEM test is now preferred as it is easier to read and more sensitive for CD without loss in specificity.[71]

ANTI-ENDOMYSIUM ANTIBODIES

Anti-endomysium (AEM) antibodies were first described by Chorzelski *et al*. in 1984.[53] They are directed against the connective tissue surrounding smooth muscle fibres [53] and are detected using an immunofluorescence technique. Positive samples give a honeycomb staining pattern in the connective tissue surrounding the myofibrils whereas smooth muscle antibodies stain myofibrils homogeneously.[53,71] Interestingly, while ARA reacts with both human and rodent tissues,[108] AEM antibodies are more species-specific and react only with the endomysium of the gastro-intestinal tract of primates.[111]

The technique was initially developed using monkey oesophagus (MO) as the substrate,[53] but due to ethical and cost reasons there has been a need for alternative substrates. In 1994 Ladinser *et al*. proposed the use of human umbilical cord (HUC) which is rich in matrix proteins surrounding the smooth muscle fibres in the wall of the umbilical vein and arteries.[112] They reported that HUC had a 100% sensitivity and specificity compared to 90% with MO suggesting that human tissue was a more sensitive substrate than MO.[112] Others have confirmed that the sensitivity of HUC is identical to[113-117] or superior to[118,119] that of MO. Although other substrates have been proposed including human oesophagus[120] and human umbilical endothelial cells (HUVEC),[121] which are of comparable sensitivity and specificity to MO, they are not in common use. Frozen sections of MO or HUC are commercially available.

AEM antibodies are predominantly of the IgA class,[53] usually IgA-1 subclass.[121] The IgG AEM test produces more false-positives and should not be used,[71] except in IgA deficiency where it has been shown to be sensitive.[36] There is no consensus as to whether AEM and ARA are in fact identical. Their overlap in sensitivity and specificity suggests that they are the same, but that the rodent tissue is not as sensitive as the primate substrate.[52,71] Others have found discrepancies between AEM and ARA results indicating that they are not identical.[81,111,115]

SENSITIVITY AND SPECIFICITY OF IGA AEM ANTIBODIES

Chorzelski *et al*. reported a sensitivity of 78.9% and specificity of 100% in 38 DH patients.[53] Since then others have found sensitivities ranging from 74-100% and specificities ranging from 96-100%.[20,36,37,80,81,112,113,115,118,119,121-126] Unfortunately, few studies have been able to reproduce the reported 100% sensitivity and specificity of AEM.[37,81,121,122] (Table 2) The PPV and NPV of AEM antibodies range from 79-100% and 95-100% respectively.[20,81,119,125,126] AEM has never been found to be positive in healthy controls and has so far been negative in disease controls (Crohn's, UC and other gastrointestinal diseases) but has been found in two asymptomatic patients with IDDM and Hashimoto's thyroiditis;[81] unfortunately both patients declined further investigation, and it is possible that they had silent CD which is more common in auto-immune diseases.[81] False-positive AEM results have also been reported in a child with cow's milk enteropathy,[127] in six symptomatic children (poor weight gain/growth, abdominal pain, vomiting) without histological abnormalities,[127] and in two children with giardiasis.[111,126] Seroconversion and histological improvement followed anti-microbial treatment.[111]

Although the AEM test is highly sensitive, it fails to detect all gluten-sensitive individuals. False-negative results have been reported in untreated CD patients on a normal gluten-containing diet after the exclusion of SIgAD.[66,114,128]

A positive AEM depends on age, the severity of the mucosal lesion, possibly on the length of intestine involved,[129] and on genetic factors.[20] About 12% of CD children under the age of two years are falsely negative for IgA AEM;[130,131] AGA is more sensitive in this age group.[19] The best diagnostic approach in children appears to be a combination of IgA and IgG AGA and AEM which gives a PPV of 99.6% and NPV of 99.3%.[130] AEM is clearly superior to AGA or ARA antibodies in selecting patients to undergo definitive intestinal biopsy.[81,85]

ANTI-JEJUNAL ANTIBODIES

Serum IgA antibodies binding to human jejunum (JAB) were first described by Karpati *et al*. in DH patients in 1986 and later in untreated CD patients in 1990.[132] They are detected using an immunofluorescent technique on cryostat sections of human fetal gut or normal human jejunum.[132] The sensitivity in DH was 72%[132] and in untreated CD was 93%,[82] with specificity of 100%.[132] (Table 2) Antibody titres did not correlate with the severity of the jejunal lesion.[132] JAB disappeared after treatment with a GFD and re-appeared in 90% of patients following gluten challenge.[82]

Like other tissue antibodies, the sensitivity of JAB falls in children under the age of two years to 71%.[82] Simultaneous determination of AGA, AEM absorption studies,[82] as well as ultrastructural studies,[133] have suggested that JAB is identical to AEM but is different from AGA.[82] Karpati has further suggested that JAB may also be identical to IgA ARA on the basis of staining characteristics.[132] Fetal jejunum has been proposed as an alternative substrate to MO because JAB are closely related to or identical to AEM,[115] but the use of fetal tissue would raise as many ethical issues as the use of monkey tissue. These antibodies are not therefore in common use.

ANTI-TISSUE TRANSGLUTAMINASE ANTIBODIES

In 1997 Dieterich *et al*. reported that the enzyme tissue transglutaminase (TTG) was the long sought after auto-antigen with which AEM antibodies interact in CD sera.[134] This finding has been confirmed by others, who have also shown that ARA antibodies may also arise due to an immune response to TTG.[135] Since then, attempts have been made to develop an objective, semi-quantitative IgA ELISA which would reproduce the subjective AEM immunofluorescent findings.

TTG is a calcium-dependent enzyme which catalyses the formation of ε–(γ-glutamyl) lysine bonds between protein-bound glutamine and lysine residues, and is widely distributed in various body fluids and tissues.[136] TTG has been implicated in many physiological settings, including in wound healing through its cross-linking activity with extracellular matrix proteins (fibronectin, collagen II, V, XI, and procollagen II).[137] TTG has very selective substrate characteristics requiring a high glutamine content.[136] Gliadin is an excellent substrate for the enzyme as it contains approximately 40% glutamine residues.[138] The amino acid sequence of TTG is highly conserved among the species and the commercially available enzyme from guinea pig liver (Sigma) shares 80% sequence homology with the human enzyme.[139] However, Sigma TTG is not pure and may vary from batch to batch.[140] Unfortunately, there is still no commercially available human recombinant form, however its use in research settings indicates that it may be superior to the guinea pig liver source.[141,142]

The sensitivity and specificity of the IgA TTG ELISA range from 85-98.1% and 94-97% respectively (Table 2);[143-145] the PPV was 92%.[145] IgG anti-TTG is not specific to CD.[134] The IgA TTG antibody concentrations correlate well with the IgA AEM titre.[144] IgA TTG is significantly higher in untreated CD than treated CD.[144] Dieterich *et al*. found four CD patients were AEM-positive, but TTG-negative, and that ten AEM-negative samples had an elevated TTG.[144] They suggested that the discrepancies between the IgA TTG and IgA AEM were due to differences between the substrates used - MO for the AEM and guinea pig liver for the TTG ELISA.[144] Like AEM, IgA TTG antibody concentrations correlate with the severity of the duodenal histology.[128] TTG and AEM were both positive in 66% of patients with STVA, but in only 50% and 20% respectively of patients with PVA.[128]

False-positive IgA TTG results have been reported in disease controls with normal duodenal histology and include: abdominal pain and dyspepsia (four), gastroesophageal reflux (four), gastritis or gastric ulcer (four), and IBS (one),[143] unexplained anaemia (one), and a CD relative (one).[145] No patients with Crohn's disease, ulcerative colitis or IDDM were positive for IgA TTG.[143] The TTG ELISA does not exactly replicate AEM findings and produces more false-positive results.

The favourable sensitivities of the IgA TTG ELISA reported need to be interpreted cautiously because of the low proportion of AEM-negative CD patients included in these studies: 10/136 (7%),[143] 1/106 (0.9%),[144] and 1/27 (4%).[145] In our experience, patients were selected to undergo duodenal biopsy on the basis of high clinical suspicion (diarrhoea, weight loss, anaemia, abdominal pain or in a high-risk group) even if the IgA AGA and IgA AEM were negative. In this setting, we found 13/53 (25%) of untreated CD patients all on a normal diet were IgA AEM-negative. Only one patient had SIgAD and was positive for IgG AGA. The sensitivities and specificities of the IgA AGA, AEM and TTG were 64% and 85%, 75% and 100%, and 66% and 95% respectively. The TTG ELISA therefore needs to be evaluated further to include the full spectrum of untreated CD patients before the AEM test can be replaced.

ANTI-CALRETICULIN ANTIBODIES

Karska *et al*. showed that monoclonal AGA cross-react with epitopes on rat enterocytes.[146] Using specialised techniques, these epitopes were shown to correspond to calreticulin. Calreticulin is a multifunctional calcium-binding protein which is widely distributed within cells.[147] It can interact with integrin receptors and has thus a potential role in extra-cellular matrix interaction.[146] Calreticulin and gliadin share similar epitopes which could be recognised by anti-calreticulin (ACR) antibodies.[146] Using an ELISA technique, a significant correlation between IgA ACR and AGA in CD patients has been demonstrated.[146] Both ACR and AGA antibodies are significantly higher in untreated CD than patients on a GFD or controls.[147] It has been suggested that AGA may play a pathogenic role in CD by cross-reacting with enterocytes and that calreticulin may be one of the CD auto-antigens.[147]

ROLE OF SEROLOGY IN MONITORING GLUTEN WITHDRAWAL AND CHALLENGE

The clinical response to a GFD precedes mucosal recovery

which may be incomplete even after two years of treatment.[148] Dietary non-compliance is a common problem, particularly in adolescence (up to 50%), whether gluten ingestion is either intentional or often inadvertent,[18] and many patients are asymptomatic.[149] Monitoring compliance is difficult as objective markers such as serology do not consistently correlate with the underlying mucosal histology and dieticians are limited to a subjective assessment. Persistently positive IgA AGA and AEM are useful indicators of continuing gluten intake, but the benefit of one over the other is still in dispute.

Serum IgA AGA titres decline on gluten withdrawal (usually within the first month),[90,126] eventually approaching that of controls,[70,74,75,99,150] but may persist in the intestine even in treated patients.[151] Asymptomatic patients on a low gluten diet (2.5-5 g/day) and normal serum AGA may still have evidence of mucosal damage in the form of raised IELs.[152] Conversely, high levels of IgG AGA may persist even with strict adherence to GFD both in adults[153] and children[74,75] despite documented healing of the enteropathy. Although AGA testing is a useful adjunct in judging a suitable time for biopsy after gluten provocation,[154] the AGA may remain negative despite an adequate challenge and histological evidence of mucosal injury.[74] Unlike the AEM, after prolonged gluten ingestion the proportion of AGA-positive individuals falls despite continuing gluten ingestion and a pathological mucosa.[150] Therefore a negative AGA titre in patients who have stopped their GFD for long periods does not exclude persistent gluten intolerance.[150]

The AEM may be less suitable for monitoring gluten withdrawal due to its subjective, qualitative reporting (negative or positive) unless multiple dilutions are performed which is too time-consuming for routine practice. If multiple serum dilutions are performed, AEM titres can be noted to decline following the start of a GFD even if the sample remains positive at a 1/5 dilution.[122] The decline usually starts within the first four to twelve months.[52] After one year of treatment with a GFD, 11% of adults and 26% of children remain AEM-positive compared to 85% and 90% respectively on a normal diet.[123]

AEM results may not correspond to histological findings as some patients may remain AEM-positive despite restoration of a normal jejunal mucosa[123,155] or are AEM-negative in the presence of persistent jejunal lesions.[124] These findings suggest that AEM and CD lesions are not strictly related to each other contrary to what has been previously suggested.[82] It also indicates that the mucosa can recover in the presence of persisting antibodies, casting doubt about the role of antibodies in the pathogenesis of CD.[155] Despite these equivocal findings, some studies show a good correlation between AEM serology and histology on a GFD,[112] and that the AEM agrees more closely to histology than does AGA.[80] Re-appearance of AEM is a reliable marker of mucosal relapse associated with gluten challenge; it may also precede the appearance of villous atrophy.[130] AEM positively may precede[20] or follow AGA positively.[122,123,130,149,150]

The IgA AGA appears to be better than AEM at monitoring the initial response to gluten withdrawal as it may become negative earlier than AEM, but AEM is more sensitive at detecting mucosal relapse (often silent) after periods of gluten intake or during gluten challenge.[20]

SEROLOGICAL MARKERS IN LATENT CD

The term 'latent' CD was first introduced by Weinstein in 1974 to describe patients with DH and normal jejunal mucosa in whom typical coeliac intestinal histology could be induced following a 20 gram gluten challenge.[45] The term is used to describe patients with a normal small intestinal biopsy on a gluten-containing diet who in the past or future have an abnormal small intestinal biopsy that improves on a GFD.[131] As the diagnosis of CD is based on the finding of characteristic mucosal histology, a normal biopsy from a patient on a gluten-containing diet is generally thought to exclude CD. It has however been shown that coeliac lesions may follow a normal biopsy.[156-158]

Patients with positive serology but normal duodenal histology are thought to represent 'false-positives', but in fact may represent latent CD. Sero-positive patients with 'normal' biopsies using conventional microscopy may have subtle abnormalities, such as raised γδ-T cell receptor-bearing IELs, detected using morphometric studies.[98,159-163] The mucosal lesion may also be patchy.[16] If these AEM-positive patients are re-biopsied after an interval of about two years (or earlier if the patient develops symptoms), about 40-65% of them will have characteristic coeliac histology.[20,156,164] Persistent AEM positively is superior to AGA in predicting future CD.[156,164] These individuals should not commence a GFD until definite histological criteria are reached.[156]

Definitions:

- Latent CD - patients who have a normal small intestinal biopsy on a normal gluten-containing diet who in the past or future have had an abnormal small intestinal biopsy which recovers on a GFD.[8]
- Potential CD - a term proposed for patients who should have the diagnosis of latent CD considered because of the presence of positive serology, morphometric studies, family history or IgA deficiency without prior evidence of an abnormal biopsy.[8]
- Silent CD - patients who have characteristic intestinal villous changes which return to normal on a GFD, without manifesting clinical symptoms.[165]

RECENT ADVANCES IN THE PATHOGENESIS OF CD

The exact pathogenetic mechanisms of immunological damage in CD remain uncertain, but there have been many recent advances. Hypotheses regarding the nature of the primary defect have evolved from the 'missing peptidase' theory, to a membrane glycoprotein defect, to a mucosal permeability defect, to the most widely accepted immunological theory.[57] The role of antibodies in the pathogenesis of CD is still uncertain and may be only an epi-phenomenon useful in the diagnosis of CD but with no direct contribution to the mucosal injury.

As early as 1984, Peters *et al.* were the first to implicate TTG in the pathogenesis of CD suggesting that TTG may facilitate gluten binding to membrane components in CD.[166] It was suggested that it might not be that a 'peptidase' is missing, but excessively expressed.[166] In 1985, the same group were the first to demonstrate an increased TTG

activity in human jejunal mucosa.[138] Animal studies later showed that the enzyme is predominantly submucosal (>85%) as opposed to epithelial (10%),[167] and that while mucosal TTG activity is increased, serum activity is reduced in untreated CD.[168]

HLA DQ2-restricted gliadin-specific T cells have been demonstrated in small intestinal biopsies of CD patients but not from non-CD controls.[169] This finding supports the belief that mucosal T cells recognise gliadin peptides in association with DQ2 molecules. *In vitro* studies have recently shown that TTG produces a selective deamidation of glutamine residues (from glutamine to glutamate) of pepsin-trypsin digested gliadin creating a protein which is more easily recognised by gut-derived T cells when presented by DQ2 molecules.[170] TTG modified gliadin increases DQ2 binding ten-fold.[170] The likely scenario is that TTG forms complexes with deamidated gliadin[170] which are recognised by mucosal gliadin-specific T-helper lymphocytes, which help TTG-specific B cells produce anti-TTG antibodies.[171] This theory would explain why anti-TTG antibodies decline after gluten withdrawal and why they are more disease-specific than AGA.[171]

Interestingly, non-enzymatic deamidated gliadin (using acid/heat-treatment) has been shown to be non-toxic to coeliac mucosa;[172] only the TTG deamidation produces epitopes which were advantageous for T cell recognition.[170] Unfortunately, Molberg *et al.*[170] did not show that this deamidation would still occur *in vitro* if lysine residues were available to bind to glutamine, which is the usual action of TTG,[173] or if physiological levels of TTG in normal or inflamed mucosa would be sufficient for this reaction.[174] It is known that deamidation only occurs in the absence of available protein-bound lysine residues,[136] and under physiological conditions, it is probable that matrix proteins containing lysine residues would be available to prevent deamidation from occurring.[173] The finding of enhanced T cell recognition of TTG-gliadin in the mucosa but not in the periphery is curious as there has been no previous evidence that the repertoire of these T cells are different in these locations.[174]

Although it had already been demonstrated *in vitro* that CD mucosa, but not controls, produce AEM antibodies, the mechanism was not known.[175] The findings of Molberg *et al*. show that the submucosa provides an ideal microenvironment for the specific TTG-mediated gliadin modification, DQ2-binding and T cell recognition.[170] This theory would also explain the genetic predisposition of the disease to the DQ2 haplotype. It is unlikely that antibodies directly contribute to the mucosal lesion, as CD is common in selective IgA deficiency[28,29] and may occur in severe hypogammaglobulinaemia.[176] However, Maki *et al*. have reported that anti-TTG antibodies interfere with cellular differentiation and may contribute directly to mucosal flattening.[177] Although there are some unresolved issues with these evolving theories, enzymatic modification of proteins appears to be a new mechanism in the breaking of tolerance and in the pathogenesis of auto-immune diseases.[170]

Summary of key points:

- The ESPGAN criteria for the diagnosis of CD requires histological evidence of small bowel villous atrophy and is supported by the presence of two/three positive serological tests. A control biopsy following treatment with a gluten-free diet is only required in asymptomatic or sero-negative patients at initial diagnosis.
- The prevalence of symptomatic CD in European countries is between 1:300 and 1:1,000 individuals, but is probably higher as classical presentations are now rare.
- CD is associated with a 40-100-fold increased risk of lymphoma. Small bowel adenocarcinoma, and oesophageal and pharyngeal squamous carcinomas may also occur.
- Selective IgA deficiency is associated with a ten-fold increased risk of CD.
- Almost all patients with dermatitis herpetiformis will have villous atrophy if sufficient small intestinal biopsies are taken.
- Anti-endomysium antibodies are currently the most sensitive and specific of the serological markers, although the anti-tissue transglutaminase antibodies show future promise.
- Serological tests should be used in combination to improve the overall sensitivity and specificity in detecting CD.
- Sensitivities and specificities of serological tests vary widely; methodological standardisation is urgently needed.
- Individuals with positive tissue antibodies (anti-reticulin or anti-endomysium) but a normal intestinal biopsy may have latent CD. These patients should continue taking a normal diet and be re-biopsied after an interval of one to two years as many later develop villous atrophy.
- Small intestinal biopsy is still essential for the diagnosis of CD. Diagnosis should not be based on suggestive symptoms or positive serology only.

DISCUSSION

Serological tests have evolved and improved over the years. They have the advantage over intestinal biopsy in providing a non-invasive indication of underlying CD, but there is a growing opinion that they are not sufficiently sensitive or specific to replace biopsy in diagnosing CD.[20,36,41,71,87,89,119,158,178] Others believe that the AEM test is sufficiently reliable to omit duodenal biopsy in AEM-positive, but not negative, individuals.[59,125] The sensitivity of serological tests is reduced by SIgAD and immunosuppressive therapy which may produce false-negative results,[71,119] and also in milder forms of mucosal damage.[66,114,128]

Although it is known that the small intestine in untreated CD is not always flat,[15] mucosal biopsy is still used as the 'gold standard' test against which the accuracy of serological tests are compared.[155] In favour of an initial intestinal biopsy is that it does provide a useful baseline in patients who have an atypical clinical course on a GFD.[89] Some patients also want repeat biopsies to document recovery[89] and this may also improve dietary compliance. However, it should be noted that even biopsy findings may be confusing as 'all that is flat is not sprue'.[17] Positive serological markers may

help to clarify the diagnosis in patients with a flat mucosa.[119]

Problems exist in the standardisation of serological methods and reference ranges. A joint working group, founded by the European Medical Research Council and the ESPGAN, are trying to develop an international protocol using robust serological methods, positive and negative standard sera and reference material.[179] It is interesting to note that their comparison of the serological findings from eight European laboratories showed more variation in the results of the objective AGA ELISA than the subjective IgA AEM immunofluorescent test which showed almost 100% concordance for all centres.[179] Standardisation of methodologies would reduce the wide variations of reported sensitivities and specificities, and permit more direct comparison of findings from different centres.

Currently, IgA AEM is the most sensitive and specific of the serological markers, but the IgA TTG ELISA shows future promise. It has been suggested that the AGA should no longer be performed,[180] but the finding of AGA-positive, AEM-negative patients would argue against this action.[36,113,115,123] The use of a combination of serological tests may be the most reliable approach. Optimistic high sensitivities of serological markers have to be interpreted carefully, as they may have been evaluated in terms of severe (flat) mucosal lesions, or alternatively a biopsy was only taken when serological markers were positive.[66,111] Sero-negative CD does occur and a small intestinal biopsy should be performed if the clinical suspicion is high.

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