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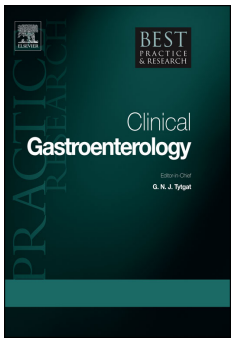

Adaptive diagnosis of coeliac disease

Ilma R. Korponay-Szabó, MD, professor, Riccardo Troncone, MD, professor, Valentina Discepolo, MD

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Authors

Ilma R Korponay-Szabó, MD, professor[1*]

Riccardo Troncone, MD, professor[2*]

Valentina Discepolo, MD[2,3]

***contributed equally**

1 Department of Paediatrics, University of Debrecen Medical School, Nagyerdei krt 98 Debrecen 4032 Hungary and Coeliac Disease Centre, Heim Pál Children's Hospital, Üllöi út 86 Budapest 1089 Hungary

2 University of Naples Federico II, Department of Medical Translational Sciences, Section of Pediatrics, Via Sergio Pansini 5, 80131 Napoli, Italy

3 University of Chicago, Department of Medicine and the University of Chicago Celiac Disease Center, Chicago 900 E 57th street, 60615 Chicago (IL), USA

Correspondence to:

Ilma R Korponay-Szabó, M.D, professor

Dept of Paediatrics, University of Debrecen Medical School, Nagyerdei krt 98 Debrecen 4032 Hungary and Coeliac Disease Centre, Heim Pál Children's Hospital, Üllöi út 86 Budapest 1089 Hungary

Phone +36-1-459-9192

Fax +36-1-459-9154

Email: Ilma.Korponay-Szabo@uta.fi

Abbreviations

CD coeliac disease

GFD gluten free diet

HLA Human Leucocyte Antigen

TG2 type 2 (tissue) transglutaminase

TGA anti-transglutaminase 2 antibodies

DGP antibodies againt deamidated gliadin peptides

EMA anti-emdomysium antibodies

ESPGHAN European Society for Pediatric Gastroenterology Hepatology and Nutrition

AGA anti-native gliadin antibodies

T1D type-1 diabetes mellitus

DH dermatitis herpetiformis

IELs intraepithelial lymphocytes

γδ gamma delta T cells

Reg3α Regenerating islet-derived 3-alpha

TCR T cell receptor

SNP single nucleotide polymorphism

PCR polymerase chain reaction

IFN interferon

Keywords: coeliac disease; gluten-enteropathy; villous atrophy; anti-endomysium antibodies; anti-transglutaminase antibodies; anti-deamidated gliadin peptide antibodies; coeliac autoimmunity; diagnostic algorhytm; case finding; biopsy-sparing; gluten challenge; dermatitis herpetiformis; type-1 diabetes mellitus; selective immunoglobulin A deficiency

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Abstract

Coeliac disease has for a long time simply been regarded as a gluten-dependent enteropathy and a duodenal biopsy was required in all patients for the diagnosis. It is now accepted that autoimmunity against transglutaminase 2 is an earlier, more universal and more specific feature of coeliac disease than histologic lesions. Moreover, high serum levels of combined anti-transglutaminase 2 and anti-endomysium antibody positivity have excellent predictive value for the presence of enteropathy with villous atrophy. This makes the histology evaluation of the gut no longer necessary in well defined symptomatic paediatric patients with compatible HLA-DQ2 and/or DQ8 background. The biopsy-sparing diagnostic route is not yet recommended by gastroenterologists for adults, and certain clinical circumstances (immunodeficiency conditions, extraintestinal manifestations, type-1 diabetes mellitus, age less than 2 years) may require modified diagnostic approaches. Coeliac patients with preserved duodenal villous structure do exist and these need a more extended evaluation by immunologic and molecular biology tools.

Introduction

The burden of diagnosing coeliac disease (CD) is high: when made, the patient has to follow a gluten-free diet (GFD) for life and, when missed, exposes the patient to elevated risk of complications which could be avoidable. Today, the symptoms are highly variable and may be even absent and the most consistent finding is autoimmunity indicated by the presence of anti-transglutaminase antibodies (TGA) accompanied in most, but not all, cases by enteropathy with villous atrophy. The diagnostic findings are gluten dependent and premature changes in the diet may interfere with the diagnosis. Nowadays, information on CD and suggestions to reduce gluten intake are abundant from the internet and other media, thus delays in appointments and diagnostic procedures may decrease diagnostic success.

Evolution of diagnostic criteria

CD was initially defined as steatorrhea on a gluten-containing diet. The first accurate diagnostic criteria were published in 1969 by the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN), which were based on three histology evaluations of the small intestinal mucosa establishing the presence of a gluten-dependent enteropathy (severe villous atrophy at diagnosis, remission on a gluten-free diet and relapse after gluten provocation)[1]. Even after the recognition of disease-specific antibodies and the reduction of required biopsies in 1990[2], for many years CD has been defined, and thus simply considered, as a gluten dependent enteropathy. However, the most recent ESPGHAN diagnostic guidelines (2012)[3] for the first time defined CD as

an immune-mediated systemic disorder elicited by gluten and related prolamines, occurring only in genetically susceptible individuals carrying the human leukocyte antigen (HLA) class II haplotypes -DQ2 and/or -DQ8 and characterised by the presence of a variable combination of gluten-dependent clinical manifestations including gastrointestinal and extra-intestinals signs and symptoms, elevated titers of coeliac-specific antibodies, autoantibodies against the enzyme type-2 (tissue) transglutaminase (TG2), endomysium and deamidated gliadin peptides (DGP) and a small intestinal enteropathy[3].

Tools for the diagnostic evaluation

Antibodies

Primary antibodies

CD is an immune-mediated disorder orchestrated by DQ2 and/or DQ8-restricted gluten-specific T cells. These T cells recognise mostly, but not exclusively, modified gliadin peptides bearing deamidation pattern specific for the enzyme TG2 and provide help for antibody production upon gluten exposure[4]. Gliadin presentation to specific T cells and anti-gliadin antibody production may occur in many people[5], but the immune response coupled with antibody production against TG2 is unique for CD. Gluten-dependent TGA are specific biomarkers of CD and they are predominatly of IgA class[6, 7]. Patients with selective humoral IgA deficiency (total serum IgA <0.05g/l) produce IgG and IgM class TGA[8, 9]. Recently, disease-specific epitopes of TGA have been identified and they show a very conservative pattern common in all CD patients[10, 11]

TGA are produced in gut plasma cells[12] and the antibodies locally bind to the TG2 autoantigen in the mucosa[13]. TGA are first accumulating in tissues expressing TG2 (gut, liver, spleen, heart, kidney, brain, endocrine glands, placenta) and also may appear in the circulation, duodenal juice[13] and saliva[14]. It is important to note that serum TGA levels reflect only the tip of an iceberg, and TGA can be produced and present in the patient's body even if serum levels are low, fluctuating, or absent at a certain timepoint. However, seronegative CD is uncommon and in such cases special caution and additional tests are needed to satisfactorily confirm the CD diagnosis[15].

TGA antibodies can be detected from blood or other body fluids by a number of immunoassays, commonly by enzyme-linked immunoassay (ELISA) or radio-binding immunoassay (RIA). Automated systems (EliA, ImmunoCAP, BioFlash) offer easier handling, higher output and daily

results with a short turnaround time. Optimal results are obtained with human TG2 antigens[6]. Immunochromatographic methods with visual reading can also be used at the bedside or point of care (rapid tests)[6]. TGA have been described in a number of other autoimmune disorders, liver diseases, heart failure, psoriasis, childhood infections with Epstein-Barr virus (EBV)[16]. In contrast to real coeliac TGA, these other antibodies are not gluten dependent, often occur in a low concentration and have different epitope specificity[10] and VH usage[17]. Despite the numerous publications mentioning these non-CD antibodies, well optimised tests detect nowadays most often real CD-associated TGA positivity, and the presence of CD must be carefully evaluated in every case with TGA positive (TGA^+) results. The coeliac relevance of a TGA^+ result can be easiest checked by immunofluorescent test in which the serum samples are added to frozen normal tissue sections containing abundant extracellular TG2 (human umbilical cord, monkey oesophagus) and the TG2-specific binding pattern is evaluated by microscope (endomysial antibody test, EMA). In other words, EMA antibodies are a special, CD-specific subgroup of TGA[18]. EMA^+TGA^+ subjects are thus defined as having coeliac autoimmunity[19]. In the EMA reaction, the TG2 protein is offered as a fibronectin-bound antigen and TGAs with hidden or intracellular epitopes may not bind. Non-CD TGA antibodies generated by tissue damage or inflammation often target the intracellular TG2 epitopes and are usually negative in the EMA test[16].

EMA^+TGA^+ serum results have very high long-term predictive value for CD[20, 21]. Studies in both children and adults showed that when TGA^+ exceeds 10 times of the upper limit of normal (10xULN) called here EMA^+TGA^{+High}, properly processed and evaluated histology specimens almost invariably show crypt hyperplastic villous atrophy (Marsh III) characteristic for CD[22-24] This high (98-100%) positive predictive value of EMA^+TGA^{+High} results has now been repeatedly confirmed in symptomatic patients[25-30] in clinical settings, and similar data have recently emerged also in T1D[31], family members[32] and asymptomatic seropositive subjects detected by screening.[33] The positive likelihood ratio of EMA^+TGA^{+High} results is around 100-200[6] and in this range the low pre-test probability has little or no effect any more. The definition of EMA^+TGA^{+High} is kit dependent. The 10xULN seems to be safe for a great number of currently available diagnostic tests[3], but may not be appropriate for all local settings[26], especially if the result calculation is not calibration curve based[21]. In such cases, the high values should be established and locally validated by utilizing histology as the reference test[26]. It is important to emphasise that villous atrophy is found in more than half of cases with EMA^+TGA^{+Low} results as well[33], thus histology evaluation is clearly recommended for this group (Table1 and 2).

The use of antibodies against native gliadin peptides (AGA) is not recommended in the diagnosis of CD due the low sensitivity and low specificity of AGA results[6]. Antibodies against DGP have better specificity[7], but surprisingly IgA DGP^+ seems less useful than IgG DGP^+ and adding DGP tests in TGA^+ patients may result in lower specificity than using TGA^+ alone.[34] IgG DGP tests have been suggested in patients with unknown total serum IgA values or IgA deficiency, and in cases with malabsorption and EMA^-TGA^- status, especially if the patient is younger than 2 years of age. Unfortunately approximatively 30% of healthy infants with a familiar risk produced DGP without TGA shortly after gluten intake in a prospective study[35] and the positivity lasted even beyond 2 years of age.

TGA, EMA and DGP IgA and IgG antibodies in CD patients are gluten-dependent, but they may decrease with different kinetics on a GFD[22]. It is thus important to check for gluten amounts in the diet when only some of these antibodies are positive and the others are negative.

Secondary antibodies

There are important inflammatory changes in the small bowel during the active phase of CD with increased apoptosis and tissue damage. Such changes can occur also in other organs, e.g. in the brain or in endocrine glands. Thus secondary antibodies against cell contituents can be generated in nonspecific ways. Antibodies against actin, DNA, immunoglobulins, Purkinje cells, thyreoglobulin or pancreatic islet cells can be found at elevated levels in active CD patients, but some may not be simply responsive to a GFD[36]. In cases with dermatitis herpetiformis (DH) antibodies reacting with transglutaminase 3[37] , in cases with neural involvement antibodies reacting with transglutaminase 6 can be present[38]. These secondary antibodies may indicate specific organ damage, but they have only a restricted role in the diagnosis of CD itself. The serum level of anti-actin antibodies seems to correlate with the presence of villous atrophy[29] and this information can be useful.

Genetic tests

Currently no genetic test is available to confirm the presence of CD, therefore genetic testing is only useful to establish a predisposition risk or help exclude CD in controversial cases.

HLA-DQ alleles

The immune reaction to gluten requires the proper presentation of peptides to T cells which can occur via HLA-DQ2 or -DQ8 molecules, having suitable pockets to accomodate negatively charged moieties of deamidated gliadins. Approximatively 30% of Caucasian normal populations carry DQ2 or DQ8, but variations of these antigens can be present in Chinese, African-American and

indigeneous Australian populations as well at a lower rate[39, 40]. So a positive DQ2 or DQ8 result does not mean the person has CD, indeed he or she can be a healthy carrier. On the contrary, absence of both DQ2 and DQ8 makes the probability of CD very low or negligible[4].

The HLA-DQ surface molecules are heterodimers consisting of an alpha chain encoded by the DQA1 locus and a beta-chain encoded by the DQB1 locus, both highly polymorphic in humans. Strings of DRB1-DQA1-DQB1 molecules are usually inherited together as conserved haplotypes in Caucasians (Table 3), with very little recombination rate during many generations. However, some variations cannot be excluded giving rise to unusual haplotype combinations which can indeed present gliadin peptides but will not be recognised as canonical full DQ2 or DQ8 heterodimers at the testing. HLA-DQ testing is performed as polymerase chain reactions (PCR) amplifying alleles by specific primers or by evaluating haplotype-associated single nucleotide polymorphism (SNPs). In recent years, technical advances in HLA-DQ typing led to the recognition of more allelic variations which may be misinterpreted as HLA-DQ2 and DQ8 negative results, especially when only one chain (either alpha or beta) gets amplified due to variations on the other chain.

HLA-DQ8 has one major haplotype while DQ2 has two major (DQ2.5 and DQ2.2) and several minor variant haplotypes (Table 3). The DQ8 and DQ2.5 encoded heterodimers can present gliadin and one copy of them is sufficient to confer risk for CD[4]. On the contrary, DQ2.2 alone only presents a different set of gliadin peptides[41, 42] and when alone in heterozygous or homozygous forms, only rarely gives rise to CD. However, when a person has on one chromosome DQ2.2 and DQ7 on the other, the translated proteins formed from the alpha chain of DQ7 and the beta chain of DQ2.2 make up a heterodimer with almost identical amino acid sequence as in DQ2.5 individuals, DQ2 in trans (with current nomenclature DQ2.2/DQ7), and it is functionally equivalent to DQ2.5[4]. Further variants of DQ2.2 and DQ2.3 (Table 3) also can produce functional DQ2 heterodimers[43] and this is not so uncommon as previously thought. Since the suggestion of performing HLA-DQ typing in EMA^{+} TGA^{+High} patients to support the biopsy-sparing diagnosis, we see these variants more often in real CD patients. Recent advances in T cell studies demonstrated that rare cases with DQ9 (resembling to DQ8) also may present gliadin peptides and become coeliac[42].

Non-HLA predisposing genes

About 50 SNP polymorphisms have been found to segregate in CD patients and other inflammatory or autoimmune disorders, such as type-1 diabetes mellitus (T1D), inflammatory bowel diseases,

psoriasis, asthma, etc. (see section on genetics) and predispose to a higher level of inflammation or more vigorous immune response to common triggers by changing expression of other genes[4].
At present, non-HLA gene results cannot be utilised in clinical diagnostics. In prospective follow up of risk persons, a high number of these polymorphic alleles (>13) increases the risk conferred by HLA-DQ alone[44].

Histology evaluation

Small bowel mucosal biopsy has been so far the cornerstone for the diagnosis of CD. A distinct pattern of abnormalities has been observed in patients on a gluten-containing diet; the features include (l) partial to total villous atrophy; (2) elongated crypts; (3) increased mitotic index in the crypts; (4) increased intraepithelial lymphocytes (IELs); (5) infiltrations of plasma cells and lymphocytes as well as mast cells, eosinophils and basophils in the lamina propria; and (6) flattened, cuboidal epithelium. These alterations are not pathognomic of CD and most of them may be seen in other entities (Table 4). Hence, it is crucial to establish the gluten dependence of the jejunal lesion. It has now become clear that, from a pathological point of view, the small intestinal enteropathy in CD may be of variable severity. A spectrum of histological signs could be present. According to the Marsh classification[45] they include 1) infiltrative lesion (more than 25 IELs /100 epithelial cells) (Marsh 1); 2) crypt hyperplasia (Marsh 2); 3) villous atrophy of variable severity (Marsh 3 a, b, c). As said, these changes, even the most severe, are not pathognomonic and should always be interpreted in the context of the clinical and serological setting[46-48]. The presence of only infiltrative changes (Marsh 1) is non-specific (only 10% of subjects presenting this pattern is coeliac), but positive serology significantly increases the possibility of CD. The count of IELs at villous tip was reported to be more specific for CD[49, 50]. Among the immunohistochemical markers one of the best predictor of CD diagnosis is the increase of intraepithelial gamma-delta (γδ) lymphocytes, but the specificity of this finding is not very high[51]. One of the drawbacks of this approach is represented by the need (for the count of intraepithelial γδ+ cells) of frozen bioptic material embedded in OCT. The recent report of the possibility of counting γδ+ cells in paraffin embedded material is particularly promising in this context[52]. The problems related to the need of frozen biopsies applies also to other more recently introduced techniques, also very valuable in identifying coeliac patients, like the detection of intestinal deposits of IgA anti-TG2. In fact, the detection of such deposits by immunofluorescence has been reported to be the best marker to identify, among potential CD patients, those who will eventually develop a gluten dependent enteropathy[53].

Lesions may be patchy[54] and in a small proportion of CD patients seem only to appear in the duodenal bulb[55]. However, interpretation of the structure in bulbar biopsies may be rendered difficult by peptic injury and distortion by Brunner glands. Recent guidelines suggest that biopsies should be taken preferably during upper endoscopy from the bulb (at least one biopsy) and from the second or third portion of duodenum (at least four biopsies)[3]. Orientation is important as only a well oriented biopsy allows for a good evaluation of the villi/crypt ratio and a correct count of intraepithelial $CD3^+$ lymphocytes[56]. Even with a correct orientation pathological interpretation is a major problem. In fact, while a good agreement has been reported for the most extreme cases (normal vs subtotal villous atrophy), the agreement is usually quite poor for mild intestinal lesions. The pathology report should include information about specimen adequacy, description of the orientation, the presence or not of normal villi or degree of atrophy and crypt elongation, villous-crypt ratio, number of IELs and a grading according to Marsh-Oberhuber[3]. The use of a standard reporting format would ensure reproducibility and comparison between reports from different pathologists.

Frontiers in diagnostic testing

With the recognition of gluten-dependent mild intestinal lesions, tests beyond conventional histology have become of help in the diagnosis of CD. Immunohistochemistry to γδ+ intraepithelial lymphocytes and immunofluorescence to detect intestinal deposits of TGA are the most specific tools (see sections on biopsy). Other strategies to detect mucosal TGA can be to measure by ELISA antibodies released in supernatants of organ culture of small intestinal biopsies[17, 57] or utilizing phage display libraries from biopsies and showing the biased use of the VH5 antibody gene family[58]. This method can be used for the rapid characterization of the anti-TG2 response in a potentially large number of subjects including asymptomatic patients whose serum antibodies may be undetectable. The organ culture has been advocated as a system to make diagnosis in difficult cases, or in cases already on a GFD which are not eligible to gluten challenge[59]. Another set of diagnostic tests is based on the demonstration of mucosal damage. Serum intestinal fatty acid binding protein (I-FABP) is a sensitive marker to study enterocyte damage, but it is nonspecific for CD[60]. Regenerating islet-derived 3-alpha (Reg3α) has been detected in the circulation of 91% of active coeliac patients and in 100% of refractory cases[61]. Serum Reg3α testing is useful for discriminating mucosal enteropathies from functional intestinal disorders.

It is well established that memory CD4 T cells specific for HLA-DQ2/8-gluten complexes are present in the small intestine of patients, but not in healthy controls. The mucosal T-cell response to gluten is a hallmark of the disease that has been hitherto unexploited in clinical work-up. Interferon (IFN)-γ-secreting T cells reactive to gluten can be detected in the peripheral blood of individuals with treated CD after a short consumption of wheat-containing food[62]. Other strategies may be used to count in the blood gliadin-specific T cells. DQ2.5-glia-α1a and DQ2.5-glia-α2 tetramer+ cells may be visualised by flow cytometry, sorted, cloned and their specificity assessed by antigen stimulation[63]. CD4+ gluten-DQ2 tetramers increase in the peripheral blood of CD patients following a short gluten challenge, distinguishing them from controls, non-coeliac gluten-sensitive subjects and treated CD patients[64].

Recent studies have indicated that T cells specific for immunodominant gluten peptides express a highly biased T cell receptor (TCR) repertoire[65]. This TCR is characterised by the frequent presence of a non–germline encoded arginine residue that has a key role in mediating recognition of gliadin determinants presented by HLA-DQ2 or HLA-DQ8, as result of a strong selective process. The presence of such TCR could represent a diagnostic tool and a predictive marker to be searched in at risk subjects.

The role of HLA and non-HLA genes in the diagnostic approach to CD is discussed elsewhere. Recently, Galatola *et al* [66] reported that a small gene expression panel from peripheral blood monocytes could discriminate between active CD and healthy controls and according to multivariate discriminant analysis the expression of five genes in intestinal mucosa accounted for 93% of the seen difference. When the same approach was applied to peripheral blood mononuclear cells the discriminant equation obtained allowed a correct classification of all CD cases and of 91% of the control samples, and, when this equation was applied to treated CD patients and to disease controls, a discrimination of 100%. These observations may open the way to a new approach to the diagnosis of CD[66].

Diagnostic approach

Case finding

According to the most recent ESPGHAN diagnostic guidelines[3] testing for CD should be offered to patients with signs of malabsorption, such as chronic diarrhoea, failure to thrive, weight loss, stunted growth or other signs such as delayed puberty, amenorrhoea, iron-deficiency anaemia, nausea or vomiting, chronic abdominal pain, cramping or distension, constipation, fatigue, recurrent

aphthous stomatitis, DH, fracture with inadequate traumas/ osteopenia/ osteoporosis, and abnormal liver biochemistry. The case finding strategy should also include subjects at risk to develop CD, such as individuals affected by other autoimmune or CD-associated disorders: T1D, Down syndrome, autoimmune thyroiditis, Turner syndrome, Williams's syndrome, selective IgA deficiency, autoimmune liver disease and first-degree relatives of CD patients. Adverse outcome of pregnancies, ataxia, neuropathies and other organ manifestations of unknown origin may be additional indications for testing in adults[3],[38].

Algorithms in children and adults

The demonstration of villous atrophy in the biopsy of the small intestine has long been considered the hallmark for CD together with clinical remission after withdrawal of gluten. ESPGHAN criteria of 1990[2] and other related guidelines[67-69] regarded antibodies (AGA, TGA, EMA), their disappearance on a GFD, and HLA compatibility only ancillary and supportive of the diagnosis. Recently ESPGHAN has revised the criteria considering histology only one of the features of CD and establishing the diagnosis on a combination of symptoms, antibodies, HLA, and duodenal histology[3]. The initial approach to symptomatic patients is now to test for IgA TGA and in addition for total IgA in serum to exclude IgA deficiency (or as alternative using IgA TGA plus direct IgG DGP testing). If IgA TGA are negative and serum total IgA is normal for age (or IgG DGP antibodies are negative), CD is unlikely to be the cause of the symptoms. TGA^{+} patients should be referred to a paediatric gastroenterologist for further diagnostic workup, which depends on the serum antibody levels. TGA^{+} patients with antibody levels <10xULN should undergo upper endoscopy with multiple biopsies. Based on the evidence of a correlation between TGA^{+} titres and degree of villous atrophy, TGA^{+} patients with antibody levels at or >10xULN, the diagnosis of CD is confirmed provided that the patient is positive for EMA antibodies ($EMA^{+}TGA^{+High}$) and positive for DQ2 or DQ8 HLA testing. A GFD is started and the patient is followed for improvement of symptoms and decline of antibodies. In the rare case of negative results for HLA and/or EMA in a TGA^{+High} child, the different possibilities for false-positive and false negative test results need to be considered (Table1-2) and the diagnostic workup should be extended including repeated testing and duodenal biopsies. In totally asymptomatic people belonging to high risk groups CD should always be diagnosed using duodenal biopsies. When biopsies are indicated, at least four fragments should be obtained from the descending part of the duodenum and at least one from the duodenal bulb. The diagnosis is confirmed by an antibody decline and preferably a clinical response to a GFD. Gluten challenge and repetitive biopsies will only be necessary in selected cases in which diagnostic uncertainty remains. The main criticisms to these guidelines are related to the variability and not

uniform quality of commercial kits for the measurement of TGA antibodies[26]. Although $EMA^{+}TGA^{+High}$ serum antibodies are recognised to predict small bowel villous atrophy, it has been suggested that cut-off limits for the definitive diagnosis must be locally validated. Also other aspects of the algorithm, need for HLA typing and for EMA confirmation among the others need to be validated in already ongoing prospective studies. Further, it should be emphasised that the biopsy sparing diagnostic route is an option to choose when all preconditions are met as set forth in Table 2, but there is always an option to choose histology evaluation if uncertainties in the performance of the actually used antibody tests or in the acceptance of the patient/parent are suspected.

In adults the same strategy has been advocated[23,30], but the majority of gastroenterologists looking after adults still recommend a duodenal biopsy before the diagnosis of CD[68]. This is advised not only because of the insufficient reproducibility of antibody tests, but also for the higher number of alternative diagnoses, sometimes very serious. Furthermore, in adults the initial biopsy could be more important for the follow-up, also in consideration of the possibility of lack of response to the GFD. However, it is an important additional point for consideration that in patient groups with high pre-test probability (e.g. malabsorptive symptoms) the positive predictive value of $EMA^{+}TGA^{+High}$ results is close to 100% whereas even when taking four biopsy samples, not more than 66% of the patients in general will have well orientated and thus easily evaluable samples[70]. So the uncertainty of the histology evaluation compared to the serology evaluation can be higher than previously thought.

In adults there is no consensus on the need of performing duodenal biopsies once a GFD is started. Most experts agree that subsequent biopsies are not mandatory if the patient is asymptomatic and has no other features suggesting risk of complications. In conclusion histology still remains mandatory for the diagnosis of CD in adults. However, the recent demonstration that EMA^{+} patients benefit from a GFD irrespective of symptoms and histology[71] suggests that serology may become the main criterium for prescribing a GFD in the future.

Clinical situations with special considerations

Patients with low serum IgA level

Selective humoral IgA deficiency (defined as total serum IgA <0.05 g/l) occurs in 1 in 500 people, is coupled to HLA-DQ2 background and confers elevated risk for CD (approximately 10%)[8]. These

patients only produce IgG TGA, EMA and DGP antibodies (or IgM locally in gut and in secretions[9]) when coeliac, but not IgA. Methods detecting serum IgA may have different sensitivity, thus it seems to be wise to search for IgG class antibodies in all patients with total serum IgA levels <0.2 g/l, even though some of these might not be really IgA deficient. IgA deficient and IgA competent CD patients do not differ either clinically or in their response to the gluten-free diet. However, the decrease of IgG class antibodies is much slower and high levels of IgG TGA and EMA positivity can persist even after 3-4 years on diet[8].

Diagnostic approach. Efficient case finding can be done by using IgA-TGA in conjunction with IgG DGP as the initial test when serum total IgA level is unknown. It is recommended to perform IgG TGA or IgG EMA determination when total serum IgA is low and/or the patient is IgG-DGP^{+}. Histology evaluation at initial diagnosis is important in patients with selective IgA deficiency, even in EMA^{+}TGA^{+High} cases.

Minimal requirements for diagnosis. Demonstration of enteropathy Marsh≥2 in conjunction with seropositivity for CD antibodies (preferably IgG EMA^{+}TGA^{+}, or at least IgG DGP^{+}). HLA-DQ testing is not helpful since non-affected IgA deficient patients usually are HLA-DQ2 positive.

Caution and pitfalls. CD may be missed if total serum IgA level is unknown and only IgA based test is used for initial evaluation[3]. It is not practical to omit initial histology evaluation in IgA deficient CD cases, because IgG EMA^{+}TGA^{+High} may persist for a long time on diet without measurable decrease[8] and this may cast doubt on CD diagnosis. Interpretation of mild inflammatory duodenal changes is difficult, because they may be associated to IgA deficiency itself.

Patients with other immunodeficiencies or immunosuppressive medications

The common problems are undetectability of coeliac antibodies and a possible villous damage even without CD due to the immunocompromised condition itself, chronic infections or graft-versus-host reaction. In common variable immunodeficiency and protein-losing enteropathy both serum IgA and IgG levels can be very low. In CD patients with severe malabsorption or with co-morbidities with food allergy, Crohn's disease, lymphangiectasia or nephrosis syndrome, the produced coeliac antibodies can be lost into the gut[20] or urine. Also patients having previously received corticosteroids, azathioprine, cyclophosphamide, methotrexate, anti-TNF alpha, antitumor drugs or preparation for bone marrow transplantation etc. may be seronegative for a variable period of time. These drugs also may influence villous atrophy and may cause false negative biopsy findings.[20]

Diagnostic approach. The emphasis is on finding some indications for TGA production either in the serum, gut mucosa or upon diagnostic gluten challenge when a temporary cause could be over.

Serum total levels of all three immunoglobulin classes should be determined. TGA antibodies may be detectable in the appropriate immunoglobulin class even at levels of 0.04-0.07 g/l of IgA or IgG or during medication with moderate doses of steroids. In autoimmune and tumour patients with TGA^+ results, the EMA test is always recommended before proceeding to small bowel biopsy. When CD is suspected clinically and serum antibodies are negative, HLA-DQ typing may be helpful to see if the patient is at risk or not. The timing of histology evaluation may be difficult and both false positive and false negative results can be common and therefore it is important to save frozen specimens and material to extended evaluation for coeliac antibody clones by more sensitive molecular biology tools. In patients with secondary immunoglobulin deficiency or after drug withdrawal, a new search for antibodies may be rewarding.

Minimal requirements for diagnosis. Demonstration of a Marsh>2 enteropathy which is clearly gluten dependent (when coeliac antibodies are not detectable, several biopsies and gluten challenge may be needed), also considering drug exposures and effects. In EMA^+TGA^+ patients with correct HLA-DQ background, CD should be considered even when histology evaluation was not conclusive.

Caution and pitfalls. These are very difficult patient groups and often no final diagnosis can be reached. Serum total IgG always has to be measured when total serum IgA is <0.05 g/l. It should be considered, that enteropathy may improve and worsen independently of gluten and moderate villous damage is common in these conditions even without CD. Signs of CD and seropositivity may 'de novo' appear after drug withdrawal. Whenever possible, it is advisable to screen autoimmune patients for CD before the immunosuppressive treatment. After bone marrow transplantation, T cells and HLA-DQ of the donor may determine the immune response. There are case reports both on the cure and transmission of CD in bone marrow transplant cases[72, 73], but it is often difficult to prove this if the recipient had been tested either before or after the transplantation during a period with drugs or shortly thereafter.

Extraintestinal organ damage

Extraintestinal manifestations can be part of the malabsorptive syndrome (osteoporosis, bleeding disorder, amenorrhea). Other organs can be affected by TG2-specific immunglobulin (mainly IgA) deposition and consequent infammation[74]. Severe liver damage, proteinuria, haematuria, myocarditis, cardiomyopathy, muscular weakness, restrictive pulmonary disease, lymphadenopathy, brain damage or ataxia can be leading problems of CD even in the absence of gastrointestinal

symptoms, or even with only mild lesions in the gut. Neural manifestations have degenerative character and are linked to the antibody response to neural transglutaminase (TG6)[38]. Extraintestinal manifestations respond to a gluten-free diet unless irreversible loss of function has already occured.

Diagnostic approach. In context of unexplained organ damage, case finding by antibody testing (TGA, EMA) is recommended, and when positive, CD diagnosis established by histological evaluation of the gut. Presence of TG2 (or TG6)-specific antibodies in the affected organ can be a proof for a direct link with CD, so when such a biopsy is planned by other indication, it is important to organise that a frozen specimen will be saved and made available for testing. Demonstration of TG2-specific IgA deposition in the gut also may be useful when villous atrophy is not present.[75]

Minimal requirements for diagnosis. EMA^+TGA^+ results and clear clinical and/or histological improvement in the affected organ in response to a GFD (when the lesion is not irreversible), coupled with the demonstration of CD in the gut.

Caution and pitfalls. An immunosuppressive treatment for the respective organ manifestation may interfere with case finding (see relevant chapter).

Dermatitis herpetiformis

DH is a special form of CD with a highly pruritic, gluten-dependent rash induced by the deposition of IgA in the subepidermal region and resulting inflammation. These deposits contain epidermal transglutaminase (TG3) and DH patients have also circulating antibodies against TG3[37]. It is unclear why certain CD patients get skin symptoms, since antibodies reacting with TG3 are also detectable in many CD patients without rash. The same patients may have malabsorptive CD without rash and DH at different timepoints of their life[76]. Ninety-five percent of DH patients are positive for anti-TG2 antibodies (TGA) and EMA, 85% have villous atrophy, while the rest have normal villous architecture with or without TG2-associated jejunal IgA deposition[22]. Thus DH represents the full spectrum of intestinal manifestations of CD. The DH eruptions respond to the GFD, albeit only after a longer time (18-24 months). A symptomatic relief can be obtained by diaphenylsulfone or dapsone, but these drugs do not treat enteropathy[77].

Diagnostic approach. Search for serum TGA and EMA is helpful in selecting patients for skin biopsy. DH is diagnosed by skin immunofluorescent study from a frozen biopsy specimen taken from the uninvolved skin adjacent to visible lesions. A DH-specific skin biopsy result also proves coeliac type gluten sensitivity, regardless whether villous damage is present or not[3]. Histology evaluation of the gut mucosa is often used to demonstrate that not only the skin is involved.

Minimal requirements for diagnosis. *S*kin immunofluorescent study demonstrating granular IgA deposition in the subepidermal region, independently from jejunal histology evaluation.

Caution and pitfalls. Patients with IgA deficiency cannot have DH. DH should not be regarded as a separate entity from CD and DH patients should be followed also by a gastroenterologist, similarly to CD. DH patients preferably should not be started on dapsone, because severe side effects (methaemoglobinaemia, kidney and liver damage) and severe flare ups after stopping the drug are common.

Type-1 diabetes mellitus

The risk of T1D is higher in CD both before and after CD diagnosis compared to the general population[78], but most patients already have clinical diabetes with definitive beta cell loss before CD is diagnosed and thus T1D is not any more responsive to a gluten-free diet. It is unclear to which extent gluten and coeliac autoantibodies contribute to the damage of the pancreas. Recently TG2-associated IgA coeliac antibody deposition has been demonstrated in the pancreas of T1D patients with undiagnosed CD[79].

CD is often subclinical in T1D patients. In different studies, 3-16% of T1D cases showed TGA^{+} or $EMA^{+}TGA^{+}$ results, but antibodies are frequently present in the serum in low concentrations, may fluctuate and may appear during follow up and years after an earlier negative serology result. Thyroid and other autoimmune manifestations also develop often with time. In children with T1D, $EMA^{+}TGA^{+High}$ serology results found by screening predicted small bowel villous atrophy independently of malabsorptive signs[31]. In T1D patients with $EMA^{+}TGA^{+Low}$ or $EMA^{-}TGA^{+}$results villous atrophy may or may not be present or can be patchy, although inflammation markers are most often elevated in the duodenal mucosa.

Diagnostic approach. T1D patients at any age are an important risk group to be screened for CD. In patients with $EMA^{+}TGA^{+High}$ serology results, the biopsy-sparing diagnostic route may be considered, as endoscopy and anaesthesia need more precautions and have elevated risk in both children and adults with T1D. In cases with confirmed $EMA^{+}TGA^{+Low}$ or $EMA^{-}TGA^{+}$ results, histology evaluation of the mucosa is recommended preferably with obtaining suitable samples for an extended evaluation (intestinal anti-TG2 deposits, morphometry, patchiness). HLA-DQ typing is not very helpful, as T1D itself is also associated with DQ2 and DQ8.

Minimal requirements for diagnosis. The diagnosis of CD can be made on the basis of seropositivity and proven enteropathy, but a GFD may be suggested to the patient in order to prevent further autoimmunity even when only mild duodenal lesions are found. Although current ESPGHAN guidelines recommend a biopsy in asymptomatic $EMA^{+}TGA^{+High}$ patients, biopsy sparing could be an option[31], according to the patient's willingness to accept the diagnosis without a histology report.

Caution and pitfalls. Postponing the biopsy and wait for persistent seropositivity has not been shown to be a safe approach as deposited antibodies and villous atrophy may persist in the gut even if serum antibody levels decline or turn to negative and thus the diagnosis of CD may be missed. T1D patients with TGA^+ results at any timepoint of their life need a careful follow up if they continue on a gluten containing diet. The significance of isolated DGP^+ results in T1D patients is still unclear.

Patients with coeliac autoimmunity and preserved intestinal architecture - Potential coeliac disease

CD has a spectrum of histological alterations, from overt intestinal atrophy up to light or absent signs of intestinal damage. This spectrum is particularly observed when the diagnosis is not solely based on the histology evaluation of the gut, but on independent variables, such as e.g. DH proven by skin immunofluorescent study (see relevant chapter). Similarly, coeliac gluten sensitive patients without villous atrophy are frequently found among those with gluten ataxia and T1D where the common denominator is the presence of TGA^+ and/or EMA^+. In the absence of other proof, TGA^+ patients with compatible HLA but without histological abnormalities in duodenal mucosa are called potential CD. The patient may or may not have symptoms and signs of the disease, and, based on the present knowledge, may or may not develop a gluten-dependent enteropathy later on. The term potential is coherent with a concept of CD where the presence of enteropathy with structural damage is essential. This is nowadays matter of debate and the same definition ESPGHAN has given of CD contemplates this condition as characterised by a variable combination of features which include, but not necessarily, the enteropathy, then envisaging cases where the histological damage is missing. Furthermore, there are reports of subjects with potential CD whose symptoms are clearly responsive to the GFD. A dietary intervention study in EMA^+ children from Finland showed that the disease was exacerbated in children who continued gluten consumption, whereas in all children who started the GFD, both the gastrointestinal symptoms and abnormal antibodies disappeared[80]. On the other hand, there are certainly cases without symptoms and others where the same conditions for the definition of potential CD are not consistent. A sizeable proportion of asymptomatic potential coeliac patients showed fluctuation or disappearance of antibody, and many of these, with persistent TGA^+ status, did not develop mucosal damage after 9 years of follow-up[81]. We still have no good way to identify which subsets of seropositive patients will go on to develop villous atrophy indicating a need for therapy. In the absence of guidelines most suggest that when a seropositive subject does not demonstrate conclusive evidence of gluten-dependent symptoms, after

extended evaluation of small intestinal mucosa, should be followed up on a normal gluten-containing diet and should be re-evaluated at regular intervals.

Patients already on a gluten-free diet and challenge procedures

Individuals with suspected but undocumented CD already adhering to a GFD cannot be accurately diagnosed, since they cannot be differentiated from healthy individuals. In this context, negative result of HLA typing may prove to be useful to (absence of HLA DQ2 or DQ8, see relevant section) help exclude CD. However, the standard of care in such subjects is to perform a "gluten challenge". The patient is exposed to gluten and monitored for symptoms, serology, possibly for noninvasive test of mucosal integrity (e.g. double sugar permeability test) and eventually for histology. Gluten challenge should be preceded by assessment of mucosal histology and should always be performed under medical supervision. Sufficient amount of gluten (around 15g/day) should be given. A recent study conducted in adults[82] has shown significant histological, serological and symptomatic changes occurring already after two weeks of gluten challenge. However, it is clear that sensitivity to gluten exposure varies greatly between coeliac patients and some may take much longer before showing signs of relapse. In adults, assessment of histology should always conclude the procedure and titres of antibodies and/or symptoms may help in deciding the right time for biopsy. In children the gluten challenge should be discouraged before the age of five years and during the pubertal growth spurt. IgA TGA (IgG in the case of low levels of serum IgA) should be measured during the challenge period[3]. The outcome of the gluten challenge procedure is considered positive (and hence the diagnosis of CD confirmed) if CD specific antibodies become positive and a clinical and/or histological relapse is observed. In the absence of positive antibodies or symptoms the challenge should be considered over after two years. However, further biopsies on a normal diet are recommended as delayed relapse may occur later in life[3].

For the future improved non-invasive markers are greatly needed. They may include markers of mucosal deterioration, but also tests assessing the presence of gluten-specific T cells[63, 64] (see section on frontiers). Finally, the possibility of in vitro gluten challenge on biopsy[59] could avoid the in vivo gluten challenge and the unacceptable symptoms it may produce which render some patients resistant to this approach.

Patients presenting at the age of less than 2 years

Patients in this age group have raised special concern for two order of reasons. The first, particularly in the past, related to possible difficulties in the differential diagnosis with other eneropathies, namely cow's milk protein intolerance. That led, at the time when ESPGHAN guidelines underwent the first revision in 1990[2], to the indication of performing gluten challenge in every child diagnosed before the age of two years. Recent studies have shown that routine gluten challenge in these children has an extremely low diagnostic yield, and it is not needed when patients have villous atrophy in combination with EMA[83, 84]. The second reason of concern has been related to the reported lower sensitivity of TGA and EMA in this age range. Although this finding has commonly been reported, in fact in most studies the sensitivity for CD of such antibodies is still around 90% (only one out of 10 coeliacs in this age group is TGA negative)[85]. Nonetheless, some have advocated for these children the routine use of DGP tests.

Diagnostic approach. The standard approach should be used also in this age range. The most recent ESPGHAN guideines suggest that, if the clincal scenario is suggestive of CD and TGA/EMA are negative, DGP antibodies should be implemented. In the biopsies obained from these patients it is still possible to look for the presence of intestinal deposits of IgA anti-TG antibodies.

Minimal requirements for diagnosis. Demonstration of enteropathy Marsh≥2 in conjunction with seropositivity for CD antibodies (IgA $EMA^{+}TGA^{+}$, or IgG/IgA DGP^{+}).

Caution and pitfalls. In the case of presentation with gastrointestinal symptoms a careful history should be taken particularly to exclude the possibility of other conditions, primarily cow's milk allergy. In this age range, as in any other case, there is no place for the measurement of serum AGA antibodies.

Conclusions

In recent years the role of antibody testing has increased while the role of histology has decreased in the diagnosis of CD, because well-controlled $EMA^{+}TGA^{+High}$ serum results can non-invasively indicate the presence of gluten-induced enteropathy. It has also been recognised that histology evaluation is not helpful in a sizeable proportion of patients due to technical problems or the variability and slowly evolving nature of the tissue damage. However, when reliable antibody results cannot be achieved in a given clinical setting or locally, the priority of performing a biopsy remains.

Practice points

- Coeliac disease may present with variable severity of symptoms and small intestinal damage
- Patients may be detected by serologic tests
- The final diagnosis must be confirmed by histology evaluation of the gut mucosa or (in symptomatic children) by the combination of HLA-DQ markers and verified $EMA^{+}TGA^{+High}$ autoantibody results indicating gluten-induced enteropathy
- There is no spontaneous cure; at present diagnosed patients should be advised to follow a lifelong gluten-free diet

Research agenda

- Natural history, predictors of deterioration and indications for treatment need to be further explored in subjects with coeliac autoantibodies but normal small intestinal stucture
- In vitro methods for assessing the response to gluten should be further developed
- There is a need for gluten non-dependent biomarkers
- Detailed studies are necessary to assess the efficacy of non-dietary treatments

Conflict of interest

IR.Korponay-Szabo declares to be a co-author in a patent application on rapid coeliac antibody testing licenced by the Univeristy of Tampere to Labsystems Diagnostics, Finland

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Table 1. Overview of diagnostic approaches at positive coeliac serology results

Serology result*	**Immediate predictive value for villous atrophy**	**Predictive value for CD during later life**	**Suggested action to take**	**Comment**
EMA^{+} TGA^{+High}	Close to 100%[22-32]	Very high	Consider biopsy sparing diagnostic route#	DGP does not add to diagnosis
EMA^{+} TGA^{+Low}	50% or more[32, 33]	Very high[80]	Proceed to histology evaluation, follow if Marsh<2 and not treated	DGP does not add to diagnosis
EMA^{-} TGA^{+High}	Variable	High, if not laboratory error	Check for recent changes in gluten intake (adopted diet?) Consider non-CD inflammatory disorders or laboratory errors§ Perform histology evaluation and consider HLA-DQ typing	CD is proven if histology Marsh≥2 DGP^{+} may be helpful
EMA^{-} TGA^{+Low}	Low[32, 86]	Low†	Perform HLA-DQ typing. Consider histology evaluation, especially if family member or T1D	DGP^{+} may be helpful
EMA^{-} TGA^{-}	Speaks against CD	Negative† [20, 86]	Check gluten intake Consider non celiac gluten sensitivity or consider other cause if villous atrophy present (verify by gluten challenge procedure) Check for TGA antibodies in biopsy tissue‡	DGP^{+} may be helpful if villous atrophy present, but DGP^{+} in children <2 years may be normal[35]

True EMA^+ TGA^- samples are very rare in untreated patients (mostly in the borderline range or gray zone), as EMA is a TGA antibody. In such cases, proceeed as with EMA^+ TGA^{+Low} cases.

[*]IgA antibodies in IgA competent cases, IgG antibodies if total serum IgA is low (<0.2g/l) or the patient is proven to be IgA deficient (total serum IgA<0.05g/l). TGA^{+High} if serum levels exceed 10 times of the upper limit of normal or at cut-off predetermined by optimization study based on histology.

[#]If clinically appropriate and additional diagnostic requirements can be met (See Husby et al[3] and Table 2). It is not practical to omit biopsy when the patient is IgA deficient (very slow antibody decrease on diet).

[§]Check for time interval between serology tests, consider sample mix-ups and inappropriate TGA cut-off, consider prozone effect at the EMA test - try to recheck with higher serum dilutions

[†]Predictive value increases if deposited TG2-specific antibodies are present in the biopsy tissue

[‡]Save frozen specimen at endoscopy in all cases with high clinical suspicion or malabsorption but negative EMA/TGA serology known before biopsy

Table 2. Requirements for choosing a biopsy-sparing diagnostic option

Obligatory components	**Practical requirements**	**Comments**
Symptoms relevant for CD	Symptoms or signs related to gut involvement and/or impaired absorption, DH proven by skin IF study. (T1D still unclear, some studies do support)[31].	Caution is recommended when only nonspecific symptoms/signs are present, e.g. constipation, recurrent abdominal pain etc. When symptoms suggest also other gastrointestinal disease, consider endoscopy and histology evaluation.
Confirmed seropositivity for CD-specific antibodies	At least two independently drawn blood samples positive	It is important to exclude laboratory or sample handling errors and mix-ups
$EMA^{+}TGA^{+High}$ result	Results by well optimized TGA kit and from reliable lab with expertise in EMA	>10xULN cut-off for high TGA results in many commercial kits, but may need to be locally adjusted based on histology results. High DGP^{+} not equivalent at present.
HLA-DQ2 or DQ8 background	HLA testing available	Prospective studies will tell if HLA testing is a significant addition in the case of $EMA^{+}TGA^{+High}$ subjects. Practical importance is that an incompatible result may draw attention to incorrect serology results. In case of non-conventional alleles, histology is required.
Evaluation and decision by a gastroenterologist	Eligible patients should be referred to a specialist	Before the final diagnosis gluten intake should not be reduced.
Acceptance from the part of the patient/family	Expert consuelling and preparation for a lifelong treatment	In case of doubts about the diagnosis either by the physician or by the patient/parent proceed to histology evaluation.

Table 3. Common HLA haplotypes associated with CD

Haplotype	DQB1 allele	DQA1 allele	DRB1 allele	Comment	Old name
DR4-DQ8	*0302	*03(01)	*04(01-11)	Heterodimer commonly presenting gliadin peptides	DQ8
DR3-DQ2.5	***02**01	***05**01	*0301	Heterodimer commonly presenting gliadin peptides	DQ2 *in cis* or DR3-DQ2
DR7-DQ2.2[87]	***02**02	***02**01	*0701	Heterodimer presenting different or restricted set of gliadin peptides[42] When DQB1*0202 is present together with DQA1*0505 belonging to haplotype DQ7, functionally identical to DQ2.5	DR7-DQ2 DQ2 *in trans*
	*0303	***02**01	*0701	Variant DQ2.2, often interpreted as DQ2 and DQ8 negative, may have CD (rare)	-
DR7-DQ2.3	***02**02	***03**03	*0701	Similar to DQ2.2, may present gliadin in combination with DQ7, may have CD (common)	-
DR11-DQ7 (DR11-DQ7.5)	*0301	***05**05	*11(01-04)	Alone probably not presenting‡ In combination with DQ2.2, forms heterodimer functionally identical to DQ2.5[41]	DQ7
DR9-DQ9	*0303	*0301	*0901	Similar to DQ8, may rarely present gliadin[88]	DQ9
	***02**02	*0301	*0901	Similar to both DQ2.2 and DQ8, possible association with CD	-

‡CD patients only exceptionally have DQ7 alone. It is possible that in those cases the other allele is a variant DQ2.2 not getting amplified or misinterpreted as a non-predisposing allele.

Table 4. Causes of small intestinal villous atrophy other than CD

Physical/Drug	**Infective**	**Immune**	**Inherited causes in enterocytes**
Mesenteric ischemia (pancreatic or duodenal surgery)[89]	Chronic bacterial overgrowth	Cow's milk protein (rarely other food) enteropathies Eosinophilic gastroenteritis	EpCAM mutation[90] (tufting enteropathy)
Irradiation	Giardia lamblia infestation	Crohn's disease in small bowel (patchy atrophy)	SPINT2 mutation[90] (tufting enteropathy plus keratitis, choanal atresia)
Cytotoxic drugs, antitumor agents[91]	Rotavirus infection	Autoimmune enteropathy[15]	MYO5B mutation[92] (microvillus inclusion disease)
Azathioprine	HIV enteropathy	Graft versus host disease	TTC37 mutation (tricho-hepato-enteric syndrome 1)
Peptic duodenitis / Zollinger-Ellison syndrome[93]	Whipple's disease	Rag2 mutation (Omenn syndrome) and other primary immunodeficiency conditions	Congenital sodium diarrhea
Olmesartan enteropathy[94, 95]	Tropical sprue	Foxp3 mutation (IPEX syndrome)	