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The Diagnostic Specialist

Coeliac Disease

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Eduardo Arranz
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Carme Farré

Presentation

This book provides a definition of the disease celiac that is as clear and concise as possible, whilst recognizing that the disorder can take many different and significant forms.

The study of celiac disease involves a wide range of practitioners including clinicians, pathologists, immunologists, epidemiologists and geneticists. Detection and treatment also concern the food industry, local government services, and health system managers.

Celiac disease is one of the most common, chronic pathologies of our time but goes largely unnoticed owing to the non-specific nature of its various clinical symptoms.

Celiac disease is the outcome of an inflammatory intestinal reaction to certain fragments or peptides of the reserve proteins of some cereals. The loss of tolerance to these proteins may occur at any age in people who generally present a genetic profile associated with HLA genes, found in around 25% of the general population.

Diagnosis through histology of a biopsy of the small intestine mucosa and treatment with a gluten-free diet were established shortly after Dicke (1950) identified dietary wheat as a factor triggering untreatable diarrhea.

Not until the late eighties were aspects of importance to the day-to-day daily lives of celiac sufferers discovered – these include the synthesis of specific antibodies (Chorzelsky 1984, Dieterich 1997), the primary association with the alleles that codify for the HLA-DQ2 molecule (Sollid 1989) and the reversible gradation of histological lesions associated with gluten sensitivity in the intestine (Marsh 1992).

However, we still lack knowledge about significant aspects such as the molecular and cellular mechanisms that determine the loss of tolerance to gliadins in genetically predisposed people; the influence of other environmental or genetic factors; how to explain the absence of clinical expression in individuals who report intestinal lesions; or the natural course of untreated celiac disease in asymptomatic patients.

The classic clinical form of the disease associated with diarrhea, abdominal bloating and malnutrition is observed in 10-20% of the total number of celiac patients. The majority have little symptomatology or show atypical clinical signs, which could partly explain delayed diagnosis in many cases.

Currently, celiac disease is not an alteration particular to childhood or exclusive to gastroenterology outpatients. The extra-digestive manifestations of the disease are multiple and varied, and celiac in patients is detected by specialists in endocrinology, hepatology, hematology, neurology, rheumatology, dermatology, gynecology, etc.

Tissue transglutaminase antibodies or serological markers of celiac disease are the main tools for detection, owing to their exceptional sensitivity and specificity. Systematic detection of these factors in individuals belonging to populations with a recognized risk greatly helps detection of patients whose clinical symptoms are not suggestive of the disease.

The irreversible secondary effects of untreated celiac disease represent the other side of the coin when considering the advisability of screening the general population.

This monograph brings together the experience of two leading professionals dedicated to the study of celiac disease: an immunologist engaged in deciphering the basic mechanisms of intestinal inflammation and immunopathogenesis of the disease, and a biochemist with many years of experience in the serological detection of celiac patients.

Eduardo Arranz
Carme Farré

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Immunopathogenesis of celiac disease. Clinical picture, treatment and therapeutic alternatives

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Clinical picture of celiac disease. Histopathology of the small intestine

Celiac disease is the most common food intolerance of our time, and is an enteropathy with varying degrees of histological lesion of the proximal small intestine. There are many factors underlying its etiology, with wheat gluten as a triggering agent along with similar proteins from cereals such as barley, rye and, to a lesser extent, oats [1, 2]. There also exists a genetic predisposition associated with genes located in the HLA-DQ region of chromosome 6 [3, 4]. The interaction between environmental factors and genetic predisposition triggers chronic inflammation and tissue remodeling of the intestinal mucosa, with changes in local immunity regulation mechanisms and loss of tolerance to gluten [4, 5]. The inappropriate adaptive immune response to gluten is mediated by specific CD4+ T lymphocytes (which infiltrate the lamina propria of the mucosa)

and preceded by changes in intraluminal digestion [6, 7], transepithelial transport of protein peptides [8, 9] and activation of mechanisms of innate immunity in the epithelium [4, 10].

Sensitivity to gluten is characterized by extensive heterogeneity of expression from a clinical, histopathological, immunological and even genetic perspective. The classic picture of malabsorption, with diarrhea, weight loss and flattening of villi of the intestinal mucosa is increasingly less common, with increased subclinical and atypical or extraintestinal symptoms, particularly in adults [1, 11]. Other environmental or local factors present when gluten is ingested (including the amount), maternal lactation, or the coexistence of gastrointestinal infections may affect immunity to gluten and the development of enteropathy, as well as explain the clinical and histopathological variability of celiac disease [12]. Several studies have estimated the prevalence of this disease as between 0.25 and 1% of the population in some western countries [1, 13-16], although only a small fraction of these cases have been diagnosed [17].

Several clinical pictures have been identified among gluten-sensitive individuals [18]: some subjects remain healthy even when gluten is present in their diet, despite their having positive genetic risk markers (potential celiac disease); some (such as direct relatives or type-1 diabetics) develop enteropathy; others are apparently healthy and show a biopsy with normal histology, but sooner or later develop intestinal lesions (latent celiac disease) or remain asymptomatic, despite showing characteristic histological alterations in the biopsy (silent celiac disease).

A high percentage of gluten-sensitive individuals show the classic pathology (active celiac disease), theoretically making diagnosis easier, since they present malabsorption, diarrhea, weight loss, flattening of the villi and positive serology. The cases most difficult to detected are those with subclinical or atypical symptoms, particularly in adults. Some have mild enteropathy (lymphocytary enteritis) and symptoms that disappear with gluten-free diets; others present immunological alterations similar to those observed in patients with celiac disease (serum antibodies, increased intraepithelial lmphocytes, gamma/delta T-cell receptors, etc.), but with poorly defined symptomatology or histology [19, 20]. Finally, there is a small group of patients with confirmed diagnosis of celiac disease, whose clinical symptoms persist after several months of treatment (refractory celiac disease) (Table I).

TABLE I. Clinical pictures of celiac disease

	HLA-DQ2/DQ8	Serology	Biopsy	Symptoms
Active, classic	+	+	+	1
Active, atypical	+	+	+	2
Silent	+	+	+	-
Latent	+	+	-	-
Potential	+	-	-	-

1: Predominantly gastrointestinal symptomatology.
2: Predominantly extra-digestive symptomatology.
Based on Ferguson A, Arranz E, O'Mahony S. Gut 1993; 34:150.

The intestine is the main target organ of gluten sensitivity. Intestinal lesions in patients with celiac disease are characterized by reversible changes that lead to restructuring of the mucosal architecture, with no tissue loss, and which disappear with a gluten-free diet. In this lesion, several interrelated stages can be recognized according to the Marsh classification [21]: Type 0 (pre-infiltrative), characterized by a mucosa of normal morphology, although local humoral immunity is altered; Type 1 (infiltrative), with normal mucosal architecture, but with infiltration of intraepithelial lymphocytes (> 30/100 enterocytes); Type 2 (hyperplastic), with extended or hyperplastic crypts and increased number of mitotic cells, but maintaining villous height and infiltration by intraepithelial lymphocytes; Type 3 (destructive), which can be partial (3a), subtotal (3b) or total (3c), and is the typical diagnosed lesion, with flattening of villi and tissue reconstruction; and Type IV (hypoplastic), a true atrophic lesion, may show collagen deposits and is observed in small groups of patients who do not respond to treatment (refractory celiac disease). Although the natural evolution of celiac disease is not known, gluten-sensitive individuals can develop any of the aforementioned types, and the degree of intestinal lesion is not related to the clinical picture (Fig. 1).

Other types of examination can be used for intestinal biopsy, in addition to the classic histopathological examination, which mainly considers the morphological aspect and architecture of the mucosa: for example,

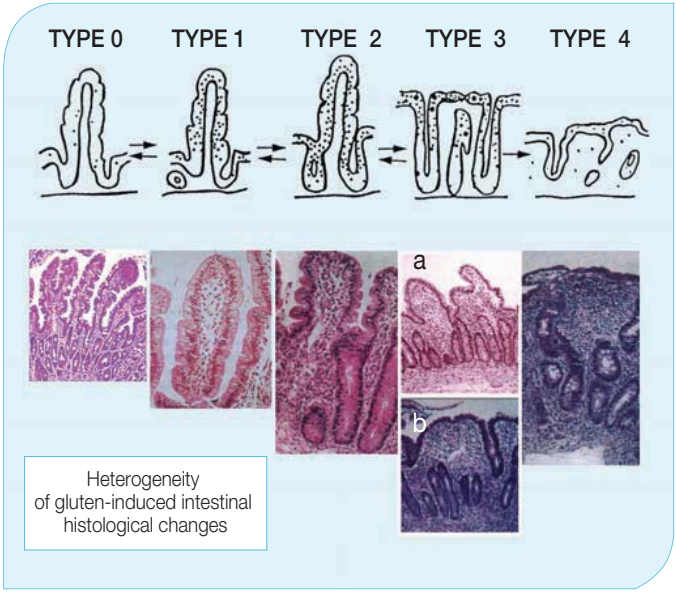

Figure 1. Marsh classification of the various types of histological lesion associated with gluten sensitivity in the small intestine mucosa.

quantitative morphometry (assesses the ratio of villous heights to crypt lengths, or the degree of cell mitosis), or immunohistochemistry (identifies certain cell populations by means of monoclonal antibodies, such as activated CD25+ cells (22), and epithelial lymphocyte (IEL) number). Isolation and phenotyping of subpopulations of epithelial lymphocytes can also be carried out using flow cytometry (epithelial lymphocytes, gamma/delta T-cell receptors, epithelial lymphocytes CD3- CD7+) (23-25).

Immunohistochemical studies have not yet been incorporated into clinical practice, probably because they involve techniques that are time-consuming, subjective and difficult to standardize. Nevertheless, analysis based on flow cytometry may be useful for identifying patients with latent and/or potential celiac disease and borderline serology, but with no flattening of villi, as well as in individuals beginning a gluten-free diet before the biopsy [23].

Immunopathogenesis: the two-signal model

At present, under the most widely accepted immunopathogenic model, gluten has a double effect, mediated both by innate immunity, with direct toxic action on the epithelium, and by adaptive or specific immunity, in which CD4+ T lymphocytes reactive for the lamina propria are involved [3, 4, 26]. Moreover, gluten ingestion induces an autoantibody response whose main target is the tissue transglutaminase enzyme [27]. Recognition by specific CD4+ T lymphocytes of native gliadin peptides and after deamidation by tissue transglutaminase in the presence of HLA-DQ2 and DQ8 molecules triggers an immune response. This response is controlled by T helper 1 cytokines – in which gamma interferon predominates [28, 29] – along with other proinflammatory cytokines (alpha tumor necrosis factor, IL-15 and IL-18 interleukins) [30, 31], but in the absence of IL-12 interleukin, while expression of immunoregulatory cytokines diminishes (IL-10, transforming growth factor beta) [31] (Fig. 2).

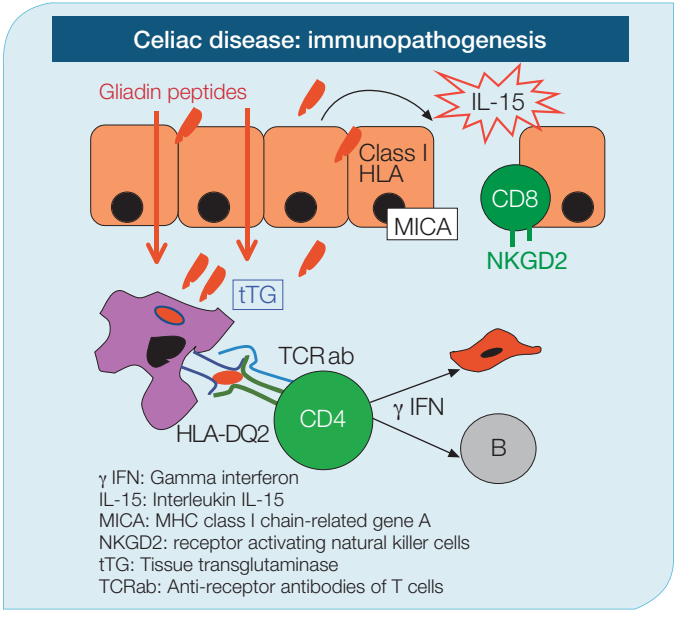

Figure 2. Gluten digestion can give rise to toxic and immunogenic peptides (T-cell epitopes). The former induce IL-15 interleukin synthesis, with increased expression of stress-induced molecules and of epithelial permeability, which allows immunogenic peptides to pass to the mucosal lamina propria. Some stress molecules (MICA) are ligands of intraepithelial lymphocyte receptors with natural killer (NKG2D) activity that may act on the epithelium. Tissue transglutaminase modifies immunogenic peptides increasing their affinity for HLA-DQ2 or DQ8 molecules. Recognition of gliadin T-cell epitopes, along with HLA-DQ molecules in the membrane of antigen-presenting cells, activates CD4+ T lymphocytes of the mucosal lamina propria, which synthesize T helper 1 cytokines (gamma interferon, IL-18 interleukin, others) and stimulate the synthesis of other local mediators.

Several elements are involved in development of the small intestine mucosa lesion in this immunopathogenic model [2, 32, 33]: the presence of gluten peptides and other prolamines (and/or their partial digestion), the direct toxic effect of some of these peptides on the epithelium, the activity of the tissue transglutaminase enzyme, the presence of antigen-presenting cells expressing HLA-DQ molecules on the membrane, and gluten-reactive CD4+ T lymphocytes, which infiltrate the lamina propria. The result of innate and acquired immune response to gluten and other similar proteins is an inflammatory lesion of the proximal small intestine with restructuring of the extracellular matrix (mucosal remodeling), which determines malabsorption of nutrients, with clinical and functional aspects dependent on the degree and extent of the lesion.

Toxic and immunogenic peptides

Wheat gluten is a heterogeneous mixture of proteins, whose two main families (gliadins and glutenins) contain fragments harmful to patients with celiac disease and are also present in proteins of rye (secalin), barley (hordein), and oats (avenin). These proteins have the generic name of prolamines because they share a very similar sequence of amino acids and a high content of hydrophobic amino acids glutamine and proline [34, 35]. Gliadins have been studied most and contain two types of peptides: *toxic* peptides, which induce intestinal lesions when added to duodenal biopsy cultures [36] or after *in vivo* administration in the proximal or distal intestine [37]; and *immunogenic* peptides (epitopes), which stimulate T-cell lines

collected from the intestine or the peripheral blood of patients with celiac disease, with HLA-DQ2 or DQ8 restriction [38, 39].

Innate or toxic peptides, like those of fragments p31-49 or 31-43 of alpha-gliadin, have a rapid effect on the epithelium and are not recognized by T lymphocytes [40]. Some immunogenic peptides are immunodominant – for example those of region 57-75 of alpha-gliadin – and induce specific immune responses in almost all patients [38, 41, 42]. Immunogenicity of peptides depends on the amount of glutamine and proline present, and their location in the primary structure, which determines molecular conformation and acts as a link for the HLA-DQ molecule, as well as determining tissue transglutaminase specificity. The main epitopes have been identified in alpha- and gamma-gliadins, but also in glutenins. Many bind to the HLA-DQ2 molecule, and others to DQ8. In the majority of cases, deamidation by tissue transglutaminase increases the affinity for the HLA-DQ2/DQ8 molecules, except in the case of DQ8 glutenins [43], which link peptide fragments to negatively-charged amino acids in central positions (4, 6, 7) for HLA-DQ2, and more external positions [1, 4, 9] for DQ8 [33, 41, 44]. Algorithms based on the separation between these residues and the presence of other amino acids have been used to identify more than 50 immunogenic peptides in gluten (gliadins and glutenins) and other prolamines, although they are almost absent in avenins [41, 44] (Table II).

The tissue transglutaminase enzyme is widely distributed in the organism and its main function is to catalyze protein

TABLE II. Examples of gluten peptides harmful to the intestine of patients with celiac disease: toxic (1) and immunogenic (2) peptides. The latter indicates the preference for HLA-DQ2 or DQ8 molecules and tissue transglutaminase enzyme activity.

Peptide (1)	Origin	Sequence
alpha-Glia (31-43)	Gliadin	PGQQQPFPPQQPY
alpha-Glia (31-43)	Gliadin	QQQPFPPQQPYPSQQP
alpha-Glia (44-55)	Gliadin	PQPQPFPSQQPY
alpha-Glia (56-75)	Gliadin	LQPFPQPQLPYPQPQLPY

Peptide (2)	Origin	HLA	TG2	Sequence
Glia (206-217)	Gliadin	DQ8	Partial	SGQGSFQPSQQN
Glt (723-735)	Glutenin	DQ8	No	QQGYYPTSPQQSG
gamma-Glia-1 (138-153)	Gliadin	DQ2	Yes	QPQQPQQSFPQQQRP
alpha-Glia-2 (62-75)	Gliadin	DQ2	Yes	PQPQLPYPQPQLPY
alpha-Glia-9 (57-68)	Gliadin	DQ2	Yes	QLQPFPQPQLPY
alpha-Glia-20 (93-106)	Gliadin	DQ2	Yes	PFRPQQPYPQPQPQ
Glt-156 (40-59)	Glutenin	DQ2	Yes	QPPFSQQQQSPFSQ
Glt-17 (46-60)	Glutenin	DQ2	Yes	QPPFSQQQQSPFSQ
alpha-Glia-30 (222-236)	Gliadin	DQ2	No	VQGQGIIQPQQPAQL
alpha-Glia-9 (57-68)	Gliadin	DQ2	Yes	QLQPFPQPQLPY

modification through transamidation or deamidation. This enzyme plays a central role in celiac disease pathogenesis by inducing deamidation of immunodominant gliadin peptides and by increasing their affinity for the HLA-DQ molecule [45],

in addition to being the main antigen of specific (auto)antibodies [27]. In patients with active celiac disease, tissue transglutaminase is expressed in the epithelial brush border and in the subepithelial zone of the lamina propria of the mucosa [46]. Gliadin is the main exogenous substrate of tissue transglutaminase. It contains positively-charged amino acids and induces the specific substitution of glutamine residues with (negatively-charged) glutamic acid residues in QXP, but not QP or QXXP sequences (Q = glutamine, P = proline, X = other) [2, 41, 44]. This mechanism may be involved in the loss of tolerance to gluten, by uncovering immunogenic epitopes, or by giving rise to other epitopes through interaction with the extracellular matrix [2, 3] (Fig. 3).

In the intestinal lumen, peptides are hydrolyzed by peptidases from the pancreas and intestinal brush border, giving rise to smaller peptides or isolated amino acids, before transepithelial transport and passage to the lamina propria, where adaptive immunity is activated. In active celiac disease, the transport of toxic and immunogenic fragments may increase [47]. Under normal conditions, incomplete intraluminal gliadin digestion is observed leading to residual fragments, such as a 33-amino acid peptide in 57-89 position of alpha-gliadin. The glutamine and proline contents of this peptide, in particular, causes resistance to enzymatic proteolysis and promotes formation of large fragments, including several immunodominant T-cell epitopes, which are tissue transglutaminase substrate of choice [6, 7, 45]. Enzymes of bacterial origin, such as prolyl endopeptidase, may induce degradation of

this fragment and prevent formation of T-cell epitopes, activators of the immune response harmful to the intestine [48].

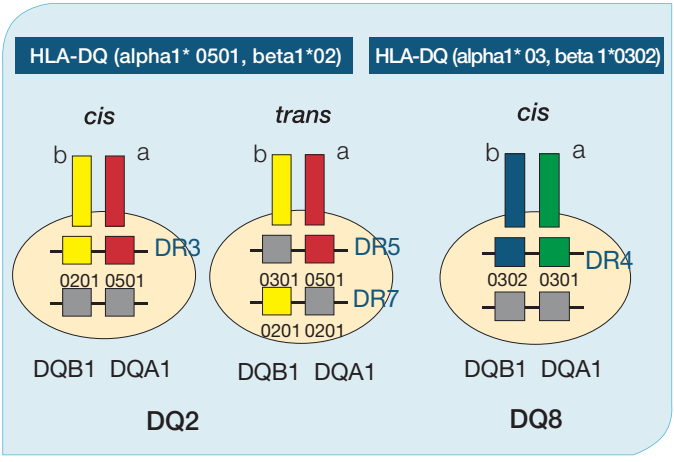

Figure 3. The HLA-DQ molecule acts as a restriction factor in antigen recognition, gluten-derived epitopes, by CD4+ T lymphocytes. Schematic representation of the interaction between a gamma-gliadin epitope and the HLA-DQ2 molecule, where links 1, 4, 6, 7 and 9 have preference for negative charges. The tissue transglutaminase enzyme induces the specific substitution of positively-charged glutamine residues (position 6 of epitope PQQPQQSFPQQRP) with negatively-charged glutamic acid residues (Q = glutamine, E = glutamic acid, P = proline).

Genetic factors associated with celiac disease

Celiac disease has a well-known genetic origin and presents one of the strongest associations with genes located in the class II HLA region, which may contribute

40% to genetic predisposition to this disease [49]. Over 95% of celiac sufferers present risk alleles DQB1*02 and DQA1*0501, or DQB1*0302 and DQA1*03 (50, 51). Negative DQ2/DQ8 cases usually have at least one of two separate risk alleles (DQA1*0501 or DQB1*02), while negative cases for both are extremely rare [52]. HLA-DQ molecules confer susceptibility, because their main function is to present small gliadin fragments (epitopes) to CD4+ T lymphocytes [2]. The associated risk depends on the quantity and *quality* of these molecules, or their capacity to link these epitopes, which is higher in HLA-DQ molecules of homozygote individuals for the HLA-DQ2.5 genotype (or DQ2.2/2.5 heterozygotes) [44] (Fig. 4).

However, the concordance for celiac disease in monozygote twins is 75%, and the frequency of DQ2 in the general population is 30%, so that only 1-2% of individuals carrying HLA-DQ2/DQ8 alleles develop celiac disease, which suggests that other factors may be involved in activation (or chronic development) of the immune response to gluten in genetically-predisposed individuals [53].

It has been suggested that various combinations of gene variants involved in immune response in each patient may determine the course and/or expression of celiac disease [2, 49]. Outside the HLA region, there are zones that contain gene candidates in chromosomes 2 (2p33: CELIAC3 [OMIM # 609755]), 5 (5q31-33: CELIAC2

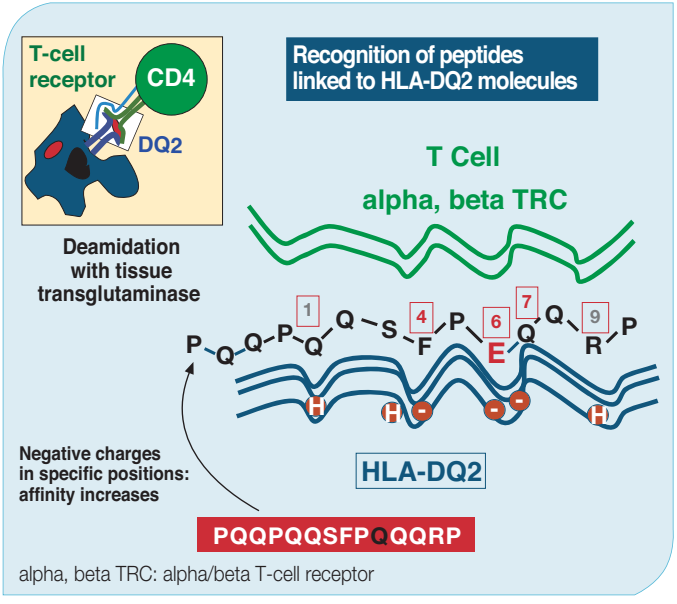

Figure 4. HLA-DQ heterodimers (a1*0501, b1*02), codified in cis position (in DR3 individuals) and trans position (in heterozygotes DR5/DR7), and HLA-DQ (a1*03, b1*0302), in cis position (in DR4 individuals), confer predisposition to celiac disease in most patients.

[OMIM %609754]), 15 (15q11-13: CELIAC5 [OMIM%607202] and 19 (19p13.1: CELIAC4 [OMIM # 609753]) [9, 54, 55]. Although the results are contradictory in celiac disease, one of these genes is CTLA4 [OMIM *123890], which is encompassed in the CELIAC3 cluster, along with CD28 [OMIM * 186760] and the ICOS [OMIM *604558]) locus.

After tracing more than 300,000 polymorphisms using

the SNP array technique (single-nucleotide polymorphisms), a recently published study [56] identified just one zone of the genome outside the HLA region that shows significant association. IL-2 and IL-21 interleukin genes are found in this zone, located in 4q27, and the authors also observe increased expression of IL-21 interleukin messenger RNA in patients with active celiac disease, compared to a control group. In a later investigation [57], which extends the study to several European DNA repositories, the same group defines several genes of molecules related to the immune system as possible risk factors for celiac disease, including CCR3 (chemokine receptor 3), interleukins IL-12A, IL-18RAP (interleukin 18 receptor accessory protein), RGS1 (regulator of G protein signaling 1), SH2B3 (SH2B adaptor protein 3) and TAGAP (T-cell activator Rho GTPase activating protein), some of which are also shared by type-1 diabetes mellitus. However, to date, only IL-21 interleukin seems to play a significant role in development of intestinal lesion [58, 59].

Innate immune response to gluten

An *ex vivo* culture model of biopsy samples from patients with celiac disease revealed that the immediate response induced by alpha-gliadin peptide 31-49 is associated with expression of IL-15 interleukin, cyclooxygenase (COX-2) and CD25 and CD83 activation markers by mononuclear cells of the mucosal lamina propria [60]. This *innate* peptide also triggers oxidative stress phenomena mediated by nitric oxide formation, which

mainly originates from inducible nitric oxide synthase (iNOS) produced by epithelial cells [61, 62], while also inducing the expression of MICA ligands for these cells [63]. In addition, gliadins can weaken tight junctions located between intestinal epithelial cells [64].

The IL-15 peptide, the main mediator of the innate response to gluten, is expressed in the celiac intestine by superficial epithelial enterocytes and mononuclear cells of the lamina propria [10, 65]. IL-15 interleukin enhances activation and proliferation of intraepithelial lymphocytes, irrespective of the interaction via the T-cell receptor (TCR). In addition it controls the clonal expansion of intraepithelial lymphocyte, gamma/delta T-cell receptors and cells with natural killer receptors (NKG2D) (66, 67), whose ligands are MICA molecules (nonclassic class I MHC) expressed by enterocytes [3, 10, 66]. Activation of cytotoxicity phenomena in the epithelium results, and this, along with weakening of tight junctions, contributes to increased intestinal permeability and passage of gliadin peptides to the lamina propria, where the adaptive immune response is triggered.

Therefore, in the immunopathogenesis of celiac disease, IL-15 interleukin would act as mediator of innate response and epithelial cytotoxicity, in addition to promoting the survival of CD4+ T lymphocytes and the maintenance of inflammatory response [68].

The direct toxic effect of gliadins on the intestine and

induced innate immune response might not be exclusive to patients with celiac disease. Stimulation of Caco-2 cell lines (enterocyte model) with gliadins leads to increased apoptosis and transepithelial permeability [69]. Moreover, gliadin has been shown to trigger dendritic cell maturation (DC) in mice as well as chemokine release [70]. In enterocyte cell lines, gliadin and 13- and 33-mer derived peptides are powerful inducers of increased zonulin-dependent [64] intestinal permeability, but also of proinflammatory gene expression and cytokine secretion in macrophage cell lines [71]. Unlike other dietary proteins, gliadin can also increase expression of maturation markers and cytokine and chemokine release in dendritic cells through a mechanism dependent on the nuclear factor kB or NFkB [72].

Adaptive immune response to gluten

Dendritic cells are the main antigen-presenting cells of the lamina propria and derive chiefly from monocytes originating from the circulation and reaching the mucosa, where they differentiate in situ in the presence of cytokines released by local T lymphocytes [73]. Dendritic cells play a vital role in local immunoregulation and can produce CD4+ T lymphocyte differentiation towards a predominantly T helper 1 or T helper 2 phenotype through a variety of mechanisms. Various intestinal inflammatory processes have also been related to changes in dendritic cells function [73-75]. Intestinal lesions in celiac disease show raised numbers of dendritic cells with HLA-DQ2+ CD11c+

phenotype, which might co-express CD123 and TLR9 (toll-like receptor 9) [76-77]. In an animal model, digested wheat gluten has been observed to induce dendritic cells maturation, which is revealed through the expression of co-stimulatory molecules and chemokine secretion by these cells [70]. However, IL-15 interleukin, the main mediator of the innate response to gluten, may also activate dendritic cells [78].

The presence of gluten-specific CD4+ T lymphocytes is confirmed in the lamina propria of the intestinal mucosa of patients with celiac disease [28]. These cells express the alpha/beta T-cell receptor (TCR) and a CD4+ CD45RO+ phenotype of immune memory B lymphocytes, and recognize gliadin peptides, such as 33-mer (56-88 of alpha-gliadin), after being modified by tissue transglutaminase in the presence of the HLA-DQ2/DQ8 molecule [38, 43].

Reactive T lymphocyte stimulation triggers a response dominated by T helper 1 cytokines, especially gamma interferon, T-bet transcription factor, and proinflammatory cytokines (alpha tumor necrosis factor, IL-18 interleukin), but without IL-12 interleukin, along with decreased regulatory cytokines (IL-10, transforming growth factor beta). This pattern disappears in patients in remission [28, 29, 31]. Transforming growth factor beta is expressed in the epithelium and lamina propria of the healthy intestine mucosa, while in celiac disease it diminishes in the epithelium and

disappears from the crypts, increasing in the lamina propria around the macrophages and activated T lymphocytes, where no tissue destruction is present [79]. IL-15 interleukin may contribute to blocking the anti-inflammatory effects of transforming growth factor beta by inhibiting the Smad3-dependent pathway [80] (Fig. 5).

In the intestine of patients with celiac disease, the absence of the main inducing T helper factor 1 (IL-12) suggests that differentiation of T helper 1 effector cells is related to other cytokines, such as alpha-interferon, IL-18, IL-21 or IL-27 interleukins, which share functions with the IL-12 molecule [30, 31, 81]. Alpha-interferon may take part in T helper 1 cell differentiation by inducing gamma-interferon synthesis. Administration of alpha-interferon to predisposed individuals has been seen to promote T helper 1 responses associated with a hyperplastic lesion [82, 83].

Moreover, in a culture model of fetal intestine, alpha-interferon antibodies block villous atrophy and crypt hyperplasia [84]. IL-18 interleukin can be synthesized by the antigen present on the cells and by epithelial cells. Unlike IL-12 interleukin, it acts on immune memory B lymphocytes and effector cells by harnessing gamma-interferon expression dependent on IL-12 interleukin or alpha-interferon. The normal intestine can express IL-18 interleukin, though an increase at the expense of its mature form is observed in celiac disease,

requiring the intervention of the IL-1 beta interleukin converting enzyme (ICE) or local proteinases [30, 31].

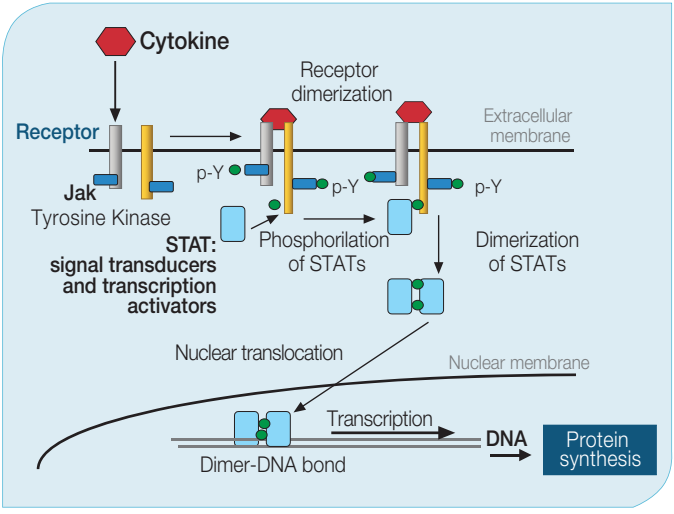

Figure 5. The interaction of cytokines with their receptors on T lymphocyte membranes activate the Janus kinase (JAK) group, which catalyze the tyrosine residues of receptors on which phosphorylation of signal transducers and activators of transcription (STAT) occurs. Phosphorylated STAT dimers can cross the nuclear membrane, binding to specific DNA sequences and activating the transcription (and expression) of cytokine genes. In celiac disease, persistent activation via STAT-1 / T-bet transcription factor may induce gamma-interferon-mediated T helper 1 responses.

In the intestinal mucosa of patients with active celiac disease, increased expression of messenger RNA of IL-27 (p28) and IL-21 [58, 85] interleukins has also been observed. IL-27 interleukin is synthesized by activated antigens

present on the cells, is made up of subunits (p28 and EBI3) similar to those of IL-12 interleukin (p45/p40), and is a powerful inducer of gamma-interferon synthesis, although it paradoxically blocks the proinflammatory action of IL-23 interleukin (81). IL-21 interleukin promotes T lymphocytes proliferation, immune memory B lymphocyte generation and natural killer cell activation (59). In celiac disease, gluten may stimulate T lymphocytes of the lamina propria to synthesize IL-21 interleukin, which would act as a mediator for gamma-interferon induction via STAT1 / T-bet transcription factor. However, it is not yet clear whether IL-21 interleukin can act as gamma-interferon inducer, along with IL-18 interleukin, or whether synthesis of gamma-interferon and IL-21 interleukin is stimulated concurrently, each of them then promoting the expression of the other, in a positive feedback circuit [59].

Interaction between cytokines and their cell surface receptors can activate different intracellular signaling pathways – such as those mediated by the group of transcription factors (*signal transducers and transcription activators*, STATs) -- modulating the expression of cytokine genes that bind to promoter regions on the gene [74]. The STAT1 pathway is activated by IL-18 and IL-27 interleukins, the STAT3 pathway by IL-6 and IL-23 or IL-27 interleukins, and the STAT5 pathway by cytokines linked to the gamma chain (IL-2, IL-9 or IL-21 interleukins).

Gliadin ingestion in predisposed individuals induces

T helper 1 responses and gamma-interferon synthesis, which leads to STAT1 persistent activation of and increased T-bet transcription factor [39, 85]. However, no changes are observed in STAT4, a transcription factor activated by IL-12 interleukin, in accordance with the observation that signaling via IL-12 interleukin is not involved in celiac disease pathogenesis. On the contrary, STAT4 is the main transcription factor in other chronic inflammatory enteropathies mediated by IL-12 interleukin [74] (Fig. 6).

This proinflammatory picture and the release of other local factors are probably responsible for the changes associated with gluten sensitivity in the intestine, resulting in mucosal remodeling. These may include keratinocyte growth factor (KGF) [86, 87], which is probably involved in crypt hyperplasia typical of celiac disease. There is also increased adhesion molecule expression in vascular endothelium and chemokine synthesis, which contribute to drawing inflammatory cells to the tissue, in addition to stimulation of matrix metalloproteinase synthesis (MMP). There is also reduced expression of tissue inhibitors of metalloproteinases (TIMP-1) [88, 89], responsible for extracellular matrix degradation and flattening of the intestinal villi. Several matrix metalloproteinases show increased expression in the inflamed intestine: MMP-1/2/3, MMP-7, MMP-9/10, MMP-12/13. In celiac disease, a correlation has been described between expression of the metalloproteinase MMP-12 and the presence of gamma-interferon with lesion extent in the small intestine mucosa [88, 90].

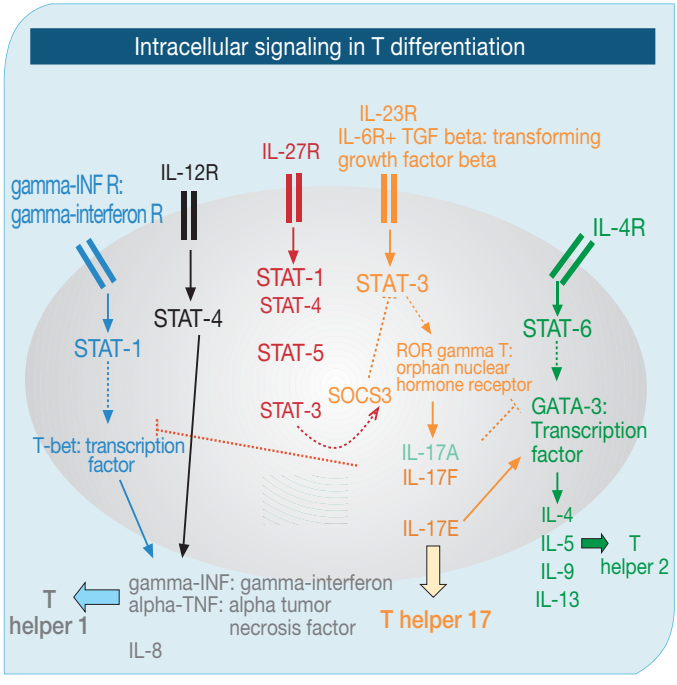

Figure 6. Illustration of the various intracellular signaling pathways activated by interaction between cytokines and their cell membrane receptors.

Treatment of celiac disease (and therapeutic alternatives)

At present, the only effective treatment available for celiac disease is a gluten-free diet, which consists of eliminating wheat gluten and similar proteins from other cereals such as barley (hordein) and rye (secalin).

Although oat toxicity is much lower, consumption is not advised because of possible contamination by other cereals during storage and transportation. Natural foods (meat, fish, eggs, milk, etc.) should be consumed, in addition to rice, maize and potatoes. Use has been made of substitutes of high nutritional value such as pulses, and some flours (e.g. those of quinoa, sorghum, millet, amaranth, buckwheat and so on). In most patients, strict adherence to a gluten-free diet leads to recovery of normal intestinal mucosal architecture, as well as symptom remission and negative serological markers.

Management is less clear in the case of asymptomatic patients, particularly those who present immunological signs of celiac disease, but no enteropathy or only minimum mucosal changes (latent and potential celiac disease) – here treatment efficacy is more difficult to assess. At the same time, one of the problems related to the gluten-free diet and establishment of safe or *acceptable limits* of gluten contents in food lies in the inaccuracy or lack of sensitivity of available quantification techniques and the lack of sound scientific data about the threshold of gluten consumption (below which there is no damage to the intestine). Recently, the risk of inadvertent gluten consumption has been evaluated in relation to the appearance of quantifiable changes in intestinal mucosa [91], suggesting that gluten ingestion should not exceed 50 mg per day.

Compliance with a gluten-free diet, however, is fraught with difficulties. These can derive from inadvertent gluten ingestion (through lack of information or wrongly identifying suitable products), but in most cases are due to voluntary transgressions, although sensitivity to gluten may vary from one patient to another. In addition, following a gluten-free diet is even more problematic in adult patients with little or no symptomatology at the time of diagnosis, or when diagnosis has been reached through a screening protocol [92]. Consuming small amounts of gluten during travel or at social events or use of treatment complementary to diet have therefore been proposed as a way of improving patients' quality of life [93].

Therapeutic alternatives

The objective of *quantitative* strategies is similar to that of the gluten-free diet: the reduction or elimination of *toxic* fragments reaching the small intestine by using wheat variants with a lower (or no) content of these peptides, or gluten detoxification using enzyme supplements. *Qualitative* strategies are based on a better knowledge of immunological and molecular mechanisms of celiac disease, and their objective is to inhibit or mitigate the immune stimulatory effects of *toxic* fragments in the intestine by blocking the activation of reactive T lymphocytes or inflammation mediators. Since celiac disease mortality is low, even though morbidity is high, and the gluten-free diet has proven effective, any therapeutic alternative has to demonstrate efficacy, safety and low cost in order to be

accepted. It should also induce good local (and systemic) tolerance, exhibit no antigenicity or unwanted side effects, and allow direct administration to the intestine [94, 95].

Quantitative strategies

In the light of current knowledge of molecular biology and plant genetics, obtaining wheat without *innate and immunogenic* peptides seems impractical and would probably lead to a modified cereal incapable of forming dough. Selecting wheat varieties with lower content of toxic prolamines seems more feasible [96]. Oral supplementation with bacterial enzymes (prolyl endopeptidases) may induce proteolysis of rich peptide fragments in proline [6, 7]. Enzymes of non-human origin have been studied by assessing their capacity to degrade peptides and intact gliadin, their stability at an acidic pH and when mixed with gastric contents, etc. However, not all gliadin peptides can be detoxified with these enzymes, and the optimal dose to obtain effective proteolysis (which may be high) is still unknown [97]. Peptidases contained in flour made from germinating cereals have also been shown to degrade toxic peptides, or proteases of certain lactobacilli can be added to fermenting dough during bread-making [98].

A recent study [99] has shown that the incubation of alpha-gliadin immunogenic peptides with tissue transglutaminase and lysine gives rise to new modified peptides that lose their binding affinity for the HLA-DQ2 molecule and consequently gamma-interferon

synthesis is inhibited. Using T-cell lines reactive to gluten from celiac sufferers, these authors demonstrate that treatment of wheat flour with transglutaminase of microbial origin (*Streptomyces mobaraensis*) eliminates its immunostimulating capacity.

By acting directly on intestinal permeability, the arrival of digested gluten peptides inside the lamina propria can be reduced, thereby improving epithelial barrier function and the structure of tight junctions between enterocytes [64, 100]. Based on observations (in an animal model) of zonulin release by epithelial cells and increased gluten-induced intestinal permeability, the use of a zonulin inhibitor (FZI/0) has been proposed for restoring epithelial integrity and preventing the transit of toxic peptides [101].

Qualitative strategies

Compounds that block the active site of tissue transglutaminase may be used to curb the formation of peptide activators of specific T lymphocytes [102]. However, this strategy has side effects due to tissue transglutaminase inhibition outside the intestine, in addition to the existence of *toxic* peptides that do not require deamidation and so may cause or maintain inflammation [41]. *Toxic* peptides may also be prevented from binding to the HLA-DQ2 molecule through competition with other analogous (synthetic) peptides, leading to functional inactivation of reactive T lymphocytes (anergy) [103].

At the same time, the use of soluble complexes formed

by HLA-DQ2 molecules and a gluten peptide would induce cell death (apoptosis) of specific T lymphocytes [104]. However, several drawbacks remain to be resolved, such as heterogeneity of known *toxic* peptides, which complicates the selection of just one of them, or the fact that HLA molecules are continuously synthesized by antigen-presenting cells and therefore functional antigenic presentation always exists [2].

The use of antagonists of the *integrin* family of adhesion molecules has been proposed, alpha4-beta7 in particular, to prevent migration of activated cytotoxic T-cells to the epithelial layer [94]. A therapy based on *alemtuzumab* (CD52 monoclonal antibody) has also been successful in a patient with refractory celiac disease [105].

Using biopsy explants of patients with celiac disease, incubation with human recombinant IL-10 interleukin was shown to curb activation of gliadin-specific T lymphocytes [105], probably as a result of a reduced stimulating capacity of dendritic cells or to reduced gamma-interferon and IL-2 interleukin levels, as well as to reduced T lymphocyte migration to the intraepithelial compartment. This finding may be therapeutically very useful if it is confirmed that T lymphocytes, isolated from the intestinal mucosa of patients with celiac disease, cultivated in the presence of IL-10 interleukin and *toxic* gluten peptides, are differentiated *in vitro* from regulatory T lymphocytes type T helper 3 or T regulatory 1 (secretors of transforming

growth factor beta or IL-10 interleukin, respectively), or if the absence of a long-term immune response (anergy) is established.

Mechanisms of tolerance induction by oral and (more recently) nasal administration of antigens exploit the generation of antigen-specific regulatory mechanisms, mainly the deletion and clonal anergy of T lymphocytes, or increased IL-10 interleukin and transforming growth factor beta synthesis, involved in regulatory T-cell differentiation. These mechanisms are complex and difficult to assess *in vivo*, and only indirect references to their function exist [4]. In a study using HLA-DQ8 transgenic mice, intranasal administration of recombinant alpha-gliadin was shown to induce diminished gamma-interferon synthesis in *in vitro* tests [106]. The result is very promising, although confirmation is required as to whether this response corresponds to what happens in the human intestine *(in vivo)*. The most suitable antigens with a tolerogenic function need to be identified and tolerance induction guidelines (dosage and mode of administration) must be established.

Blocking cytokine action has also been proposed by using, for example, gamma-interferon specific antibodies: this may be useful in celiac disease for modulating gluten response in the intestinal mucosa or the neutralizing IL-15 interleukin antibodies, which could prevent FAS (TNFRSF6) expression in the epithelium. Phenomena of enterocyte apoptosis are thereby reduced [69], or expression of alpha

chain of IL-15 interleukin receptor is induced, which in turn produces a functional block of IL-15 [10, 60]. Other molecules, for example NKG2D natural killer cell activation receptor antagonists, would also block phenomena of epithelial apoptosis [66, 67]. In addition to the complex system of cytokines, increased expression of matrix metalloproteinases, MMP-1, MMP-12 (metalloelastase) and TIMP-1 (tissue inhibitor of metalloproteinases), and diminished MMP-2 (gelatinase, prevalent in normal mucosa) have been observed in the intestinal mucosa of patients with active celiac disease. MMP-12 expression is correlated with gamma-interferon levels and with the extent of mucosal lesions [90]. These results have led to agents that inhibit matrix metalloproteinases being included as possible candidates in the treatment.

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Celiac disease

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Background

The first description of the disease dates back to the first century CE and was made by Aretaeus the Cappadocian, a contemporary of the Roman doctor Galileo. In the original text, Aretaeus referred to intestinal problems using the word *koliakos* – as a precursor to celiac – derived from *koilia*, which means belly [(1)].

In 1888, Samuel Gee, an English doctor, made the first precise and detailed clinical description of classic celiac disease as we know it today. In his paper, Gee emphasizes the fact that celiac disease affects people of all ages, advises the reduction of flours in the diet, and adds that *"if the patient can be cured at all, it must be by means of diet"*.

In 1908, pediatrician C. Herter published the first book

on the disease in pediatrics. Celiac disease came to be known as Gee-Herter's disease, and his diet for treating it was used until the middle of the last century.

In 1950, a fundamental discovery for the history of the disease was made: gluten, a protein found in wheat, was identified as the triggering factor.

Dutch pediatrician Dicke [2] observed a reduction in celiac sprue and linked it to the scarcity of cereals and bread during World War II. In contrast, when Sweden supplied bread to Holland, celiac patients suffered a relapse, so revealing the harmful effects of wheat in their diet.

In 1954 Dr J.W. Paulley discovered intestinal atrophy in a celiac patient during surgery, and this lay the foundations for diagnosis of the disease.

In 1960 the Watson-Crosby capsule became available, allowing duodenal biopsies via oral route.

In 1969 the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) gathered in Interlaken [3] to establish the first diagnostic criteria, based on three intestinal biopsies: the first at the time of clinical suspicion, the second after a period of time on a gluten-free diet, and the third after stimulation with gluten.

In 1980 ESPGAN recommended delaying gluten

introduction into the diet until six to nine months of age to avoid severe clinical pictures in future celiac patients.

In 1990 ESPGHAN (European Society of Pediatric Gastroenterology, Hepatology and Nutrition, formerly ESPGAN) reduced the diagnostic criteria to two requirements [4]: the practice of an intestinal biopsy at the time of clinical suspicion and symptom remission with gluten-free diet, reserving the use of additional tests for borderline cases.

In 1981 Unsworth described gliadin antibodies. Gliadin antibodies are the first serological markers for celiac disease and are detected as a first step prior to intestinal biopsy.

In 1984 Chorzelsky [5] described endomysial antibodies, directed to an unknown antigen, which prevail because they provide a highly effective diagnosis.

In 1989 L. Sollid [6] described primary association between celiac disease and class II HLA molecule (human leukocyte antigen), by assuming that celiac disease is a genetic alteration caused by the presence of HLA-DQ2-mediated antigen presentation, codified by genes located in the short arm of chromosome 6.

In 1992 pathologist M.N. Marsh [7] described in detail the reversibility of intestinal histological lesions in patients with celiac disease.

In 1997 Dieterich [8] identified transglutaminase, a ubiquitous tissue protein, as the antigen recognized by endomisial antibodies, which plays a key role in the etiopathogenesis of celiac disease. On the one hand, it deamidates gliadin peptides so that they are recognized by DQ2-mediated antigen presentation, and on the other, tissue transglutaminase binds to gliadin peptides, which activate CD4+ T lymphocytes, while B lymphocytes synthesize antibodies to tissue transglutaminase.

In 2002 Shan [9] identified a 33-amino acid gliadin peptide as the leading inflammatory response to dietary gluten in patients with celiac disease.

Knowledge acquired over the last 15-20 years has made a vital contribution to our understanding of the disease etiopathogenesis, and thus to improvement in diagnostic choices.

Epidemiology

The estimated prevalence of celiac disease [10, 11] in the western population is around 1%, ranging between 1.87% (1:53) and 0.15% (1:658), according to various serological studies using histological confirmation. This considerable spread reflects a real difference in risk between populations, but it is also influenced by study design, serological screening strategy, antibodies used and the criterion set for histological diagnosis in intestinal biopsy.

Prevalence studies in the general population show that celiac disease is a much under-diagnosed alteration, owing to high numbers of patients with unconventional clinical pictures, and it is estimated that clinical symptoms are identifiable in just one in every 7-10 patients. The disease is less common in men than in women, with a ratio between 1:2 and 1:3.

The celiac iceberg (Fig. 1), an epidemiological illustration introduced by Logan [12], describes the clinically identifiable cases of the disease as the *tip of the iceberg*: the underwater bulk contains a vast celiac realm of unconventional clinical pictures of the disease.

Celiac disease is a common, under-diagnosed, treatable alteration that has effective serological markers, mainly in the pediatric population. Even so, recommendations of today's

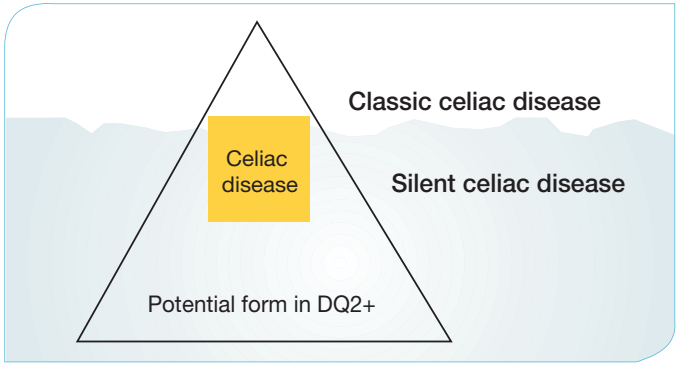


Figure 1. The celiac iceberg.

scientific bodies do not support systematic serological screening in the general population.

The main arguments against mass screening are as follows:

- Lack of studies of the cost-benefit ratio.
- Difficulty of putting asymptomatic patients on a gluten-free diet.
- Lack of knowledge about the natural course of the disease in asymptomatic patients.
- Lack of knowledge about the ideal age for screening.
- Need for regular serological studies to detect delayed gluten sensitivity.

The irreversible adverse effects of untreated celiac disease include autoimmune stimulation, osteoporosis and malignancy.

Whether or not the risk of additional autoimmune diseases such as type-1 diabetes or thyroiditis can be avoided in celiac patients by early initiation on a gluten-free diet is unknown. Studies are retrospective and too few to determine the extent to which the coexistence of organ-specific autoimmune diseases is genetically predetermined [14].

Osteoporosis brought on by inadequate bone mineralization in puberty or in adulthood is difficult to treat, and constitutes one of the principal health problems involving loss of quality of life in undiagnosed or untreated celiac disease.

Deficient bone mineralization or osteopenia found in the early years of a celiac patient is reversible through a gluten-free diet and does not generally require bone mineral density testing.

According to recent studies [15, 16], the risk of malignancy associated with untreated celiac disease is lower than previously suspected. Intestinal enteropathy-associated T-cell lymphoma (EATL), with poor prognosis and associated with celiac patients, is very uncommon, representing only 0.3% of lymphomas [17].

Clinical pictures

The classic clinical picture of the disease appears at around two years of age, several months after introduction of gluten into the diet, with the following symptoms:

- Diarrhea.
- Abdominal bloating.
- Failure to thrive.
- Irritability and vomiting.

The amount of gluten in the diet or the duration of breastfeeding may be related to the onset age of the disease. Gastrointestinal symptoms in older cases are:

- Diarrhea.
- Abdominal pain.
- Flatulence.
- Weight loss.
- Constipation.

Although celiac disease is primarily an intestinal alteration, it may appear with a wide range of other signs and symptoms unrelated to the digestive tract. Extra-digestive manifestations include:

- Short stature.
- Slight elevation of serum transaminase activity.
- Iron-deficiency anemia with no apparent cause.
- Osteopenia or osteoporosis.
- Alterations in dental enamel.
- Delayed puberty.
- Vitamin E or K deficiency.
- Hypocalcemia or vitamin D deficiency.
- Hypoproteinemia.

The clinical suspicion of celiac disease may require an intestinal biopsy in individuals at risk, such as first-degree relatives of celiac patients or patients with autoimmune diseases, independently of the result of testing for serological markers.

The diagnostic algorithm [19] proposed in Fig. 2 is consistent with scientific body guidelines and should be flexibly interpreted case by case, as with all diagnostic algorithms.

Serological markers

Celiac disease detection is mainly serological, although final diagnosis is obtained by histology of intestinal biopsy specimens.

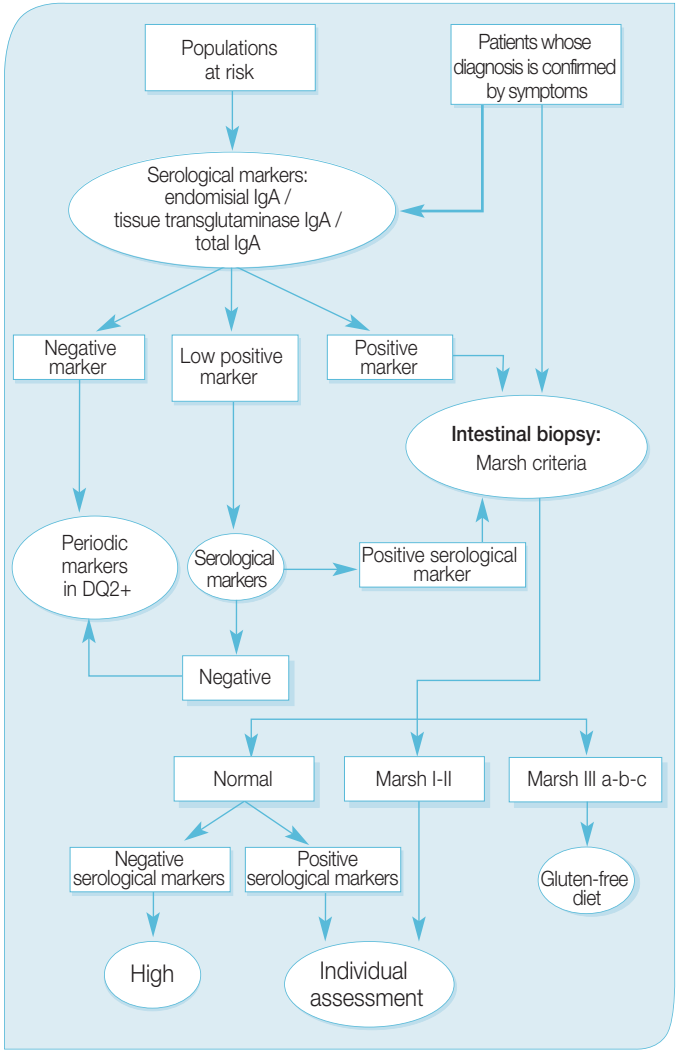


Figure 2. Diagnostic algorithm of celiac disease.

In the late 1980s, the availability of gliadin antibodies allowed identification of patients without conventional clinical pictures as a first step prior to intestinal biopsy, the only existing resource then for disease detection. The presence of gliadin antibodies of IgA class at that time was of paramount importance, and detection of celiac patients was greatly improved with the aid of endomisial IgA antibodies, to the extent that the number of annual diagnoses in 2000 was ten times that of the 1990s (Figs. 3 and 4).

Endomisial antibodies – sensitive and specific markers for celiac disease – were described by Chorzelsky (1983) in a dermatology journal because of their presence in patients with herpetiform dermatitis.

Endomisial antibodies are determined by indirect immunofluorescence, using sections of the lower third of monkey esophagus as an antigen fixed on a slide. Sections of human umbilical cord (HUC) have also been used. The microscope image corresponds to a pattern of reticular fluorescence (Fig. 5) around smooth muscle fibers. This image is similar in the esophagus and in HUC.

The sensitivity and specificity of gliadin IgA, tested using an automatic quantitative ELISA technique, are lower than those of endomisial IgA (or HUC antibodies), whose main drawback is that they are determined by a manual, qualitative technique of subjective interpretation and therefore subject to error.

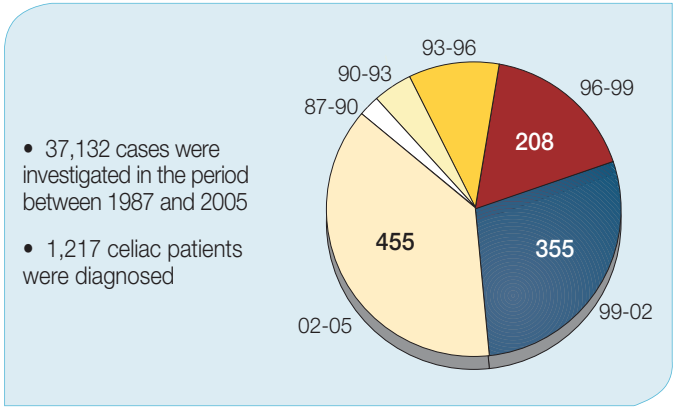


Figure 3. Serological markers of celiac disease.

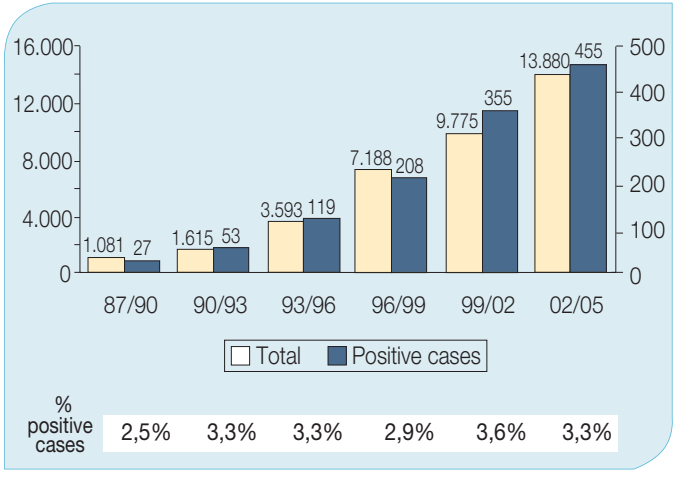


Figure 4. Total number of cases investigated versus number of patients detected with celiac disease.

In 1997 Dieterich described the tissue transglutaminase. This is a ubiquitous enzyme with various physiological functions, and is identified as the target of the humoral immune response in celiac patients. Transglutaminase antibodies are specific for celiac disease, synthesized in the lamina propria of the intestine, and detected in the blood as IgA class circulating antibodies.

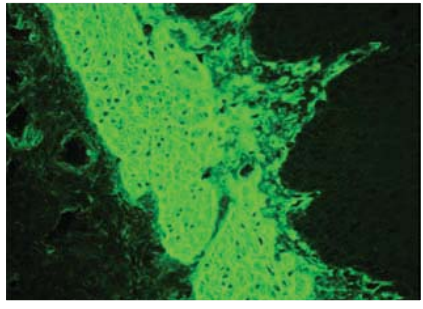
Tissue transglutaminase is very largely responsible for the enzymatic deamidation of gliadin peptides as a first step before they are recognized by the HLA-DQ2 molecule.

Obtaining either tissue transglutaminase purified from red blood cells or recombinant human tissue transglutaminase facilitates immunoassay for determination of tissue transglutaminase antibodies. These latter combine the advantages of sensitivity and specificity of endomisial antibodies (or anti-HUC) with those of an automated, quantitative and objective technique.

On today's market reagents are available for determining gliadin antibodies, endomisial antibodies and tissue transglutaminase antibodies of IgA and IgG class. Faced with the demand for serological markers of celiac disease, some laboratories use combinations of a number of these antibodies. The most recent guidelines of the North American Society for Pediatric

Gastroenterology, Hepatology and Nutrition (NASPGHAN, 2005) for the serological study of celiac disease are as follows:

- Assay of tissue transglutaminase antibodies (and/or endomisial antibodies) of IgA class and of total serum IgA concentration.
- Use of gliadin antibody is not recommended because it is non-specific and lacks of sensitivity.



Gliadin antibodies (AGA), Unsworth 1981

Endomysial antibodies (AEA), Chorzelsky 1984
Jejunal antibodies (JAB), Kärpaty; Lancet 1990
Human umbilical cord antibodies (HUC), Ladinser; Gut 1994

Transglutaminase antibodies (Anti-tTG)
Dieterich; Nature Medicine 1997

Figure 5. Serological markers.

Serological study must take place before the partial, temporary or total exclusion of gluten from the diet. The effect of gluten in the diet on serology or intestinal histology is unpredictable, causing delay and reducing diagnostic reliability.

For correct interpretation of tissue transglutaminase IgA antibodies results, laboratories should optimize the manufacturer's reference values, which are generally too broad, adjusting them to the population investigated, their experience and their own needs. Optimized reference values give a new gray area of borderline results between negative results and the lower limit of the positive results.

In our experience, the serological detection of dietary transgressions depends on individual sensitivity to dietary gluten. Some negative results for antibodies do not rule it out, whereas some positive or borderline results must be assessed in relation to the previous results of the same patient.

In patients with selective or isolated IgA deficiency, tissue transglutaminase and/or endomisial antibodies of class IgG should be determined. The reasons for recommending simultaneous detection of total serum IgA concentration are:

- 5.5% (9/162) of people with selective or isolated IgA deficiency in our geographical area suffer from celiac disease [20]. Most of these patients have been identified through systematic serological investigation with IgG

antibodies in IgA deficient individuals.

- IgA deficiency affects 0.2% of the general population and can go clinically unnoticed.

Testing for serological markers of celiac disease is recommended in the following cases:

1. Individuals who show digestive symptoms suggestive of celiac disease. These symptoms vary with age. Classic symptoms in children up to two years of age such as diarrhea, abdominal bloating, anorexia, abdominal pain, failure to thrive, and vomiting are in themselves sufficient to require intestinal histological investigation, irrespective of serology results. In older children, symptoms can vary, and include nausea, abdominal pain, flatulence or constipation. The most common digestive forms in adults are diarrhea and abdominal pain.
2. First-degree relatives of celiac patients.
3. Patients with extra-digestive symptoms related to celiac disease. Patients with herpetiform dermatitis have intestinal lesions characteristic of celiac disease and are candidates for intestinal biopsy. Patients with the following should undergo serological investigation: alterations in final tooth enamel, osteopenia or osteoporosis, short stature with no apparent cause, delayed puberty, iron-deficiency anemia with no apparent cause and mild hypertransaminasemia with no apparent cause.
4. Patients with diseases associated with celiac disease, including type-1 diabetes mellitus, thyroiditis, Down syndrome, Turner syndrome and selective or isolated IgA deficiency.

The frequency of patients with celiac disease in these populations is higher than random.

The benefit of a gluten-free diet is evident when digestive or extra-digestive symptoms related to celiac disease are presented.

However, the most recent position of NASPGHAN (North American Society for Pediatric Gastroenterology, Hepatology and Nutrition) is to advise against systematic screening in asymptomatic patients with type-1 diabetes mellitus [21, 22], arguing that the benefit of preventing possible long-term adverse effects, such as malignancy, osteoporosis or autoimmune stimulation, has not been sufficiently demonstrated. Prospective studies are needed to compare evolution in asymptomatic diabetics - detected by screening - as opposed to untreated patients. In other words, the natural course of asymptomatic celiac disease is unknown. Furthermore, it is clearly difficult for type-1 diabetes mellitus patients to adhere to a gluten-free diet, as the basic disease already complicates their diet.

The sensitivity and specificity of antibodies related to celiac disease must be interpreted with caution, since they are influenced by study population characteristics, technical aspects and histological criteria applied during diagnosis.

Table I summarizes the data published in the latest

TABLE I. Efficacy of the main serological markers of celiac disease (NASPGHAN, 2006)

	Sensitivity %	Specificity %
Gliadin IgA	52-100 in children 65-100 in adults	92-97 in children 71-97 in adults
Gliadin IgG	n.d.	~ 50 in children and adults
Endomisial IgA	88-100 in children 87-89 in adults	91-100 in children 99 in adults
Tissue transglutaminase IgA	92-100 in children and adults	91-100 in children and adults

revision [23] by NASPGHAN. According to its authors, gliadin IgA shows the lowest figures for sensitivity and specificity, whereas the diagnostic effectiveness of tissue transglutaminase IgA is similar to or slightly higher than that of endomisial IgA.

Gliadin IgA scored negative in 19 of the 31 relatives diagnosed with celiac disease in the presence of endomisial antibodies and histological confirmation detected in a multicenter study [24] carried out at nine hospitals in Catalonia (Spain) to investigate the prevalence of celiac disease in first-degree relatives of celiac patients. Without endomisial antibodies, only one third of the relatives with celiac disease would have been diagnosed.

At present, celiac disease can be effectively diagnosed

and followed up by serological markers (tissue transglutaminase and/or endomisial antibodies). Overconfidence in these markers can delay the diagnosis of symptomatic patients with negative serology. Moreover, the real size of this group of patients is unknown.

Of the pediatric celiacs in our population with negative serology and classic clinical symptoms, 13 out of 18 were younger than two. In three of them, stimulation with gluten caused seroconversion to tissue transglutaminase antibodies, but months or years later, demonstrating that the absence of specific antibodies can be transitory in children under two [25].

For this reason, some authors recommend using gliadin IgA for under-twos, referring to the work published by Bürgin-Wolff [26]. In our experience, the gliadin IgA result does not contribute additional information to that derived from the patient's clinical assessment when faced with a symptomatic child who scores negative for specific antibodies.

Celiacs with negative serology form a little-studied population, for obvious reasons, in which detection is mainly clinical, diagnosis is histological, and the appropriate response to the gluten-free diet is of paramount importance for confirming diagnosis. The absence of specific antibodies need not be a reason to avoid intestinal biopsy in patients of any age on an uncontrolled diet.

Sensitivity of tissue transglutaminase and/or endomisial antibodies is highest in patients with intestinal atrophy (Marsh III). However, sensitivity of specific antibodies can fall to values below 50% in patients with smaller lesions (Marsh I or Marsh II). In these cases, final diagnosis must be supported by HLA-DQ2 study and the clinical and/or histological response to a gluten-free diet.

Histological diagnosis

Celiac disease is diagnosed through:

1. The histological investigation of intestinal biopsy applying the Marsh classification, modified by Rostami [28].
2. The histological and/or serological and/or clinical response to a gluten-free diet.

Intestinal biopsy continues to be the test that provides evidence for final diagnosis, even though the presence of serological markers is a reason to suspect celiac disease.

Figures 6 and 7 show the reversible stages of histological lesions:

- **Marsh I**: increased intraepithelial lymphocytes. Up to 20-25 intraepithelial lymphocytes per 100 enterocytes is considered normal.
- **Marsh II**: increased number of intraepithelial lymphocytes, with crypt hyperplasia.
- **Marsh III**: increased number of intraepithelial

lymphocytes, with crypt hyperplasia and (a) moderate, (b) subtotal and (c) total villous atrophy.

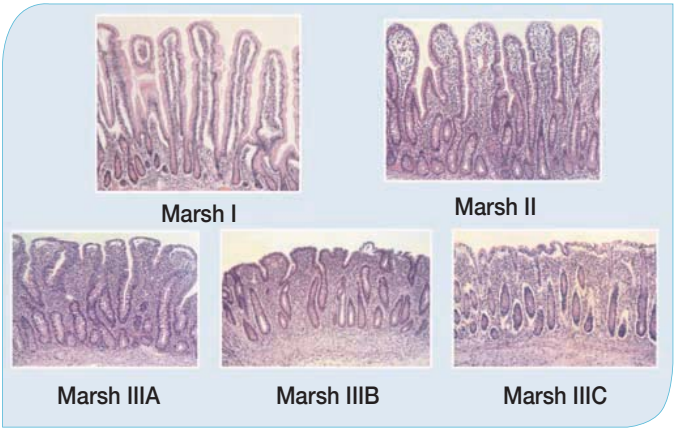


Figure 6. Degree of histological lesion in intestinal biopsy (Wahab PJ 2002).

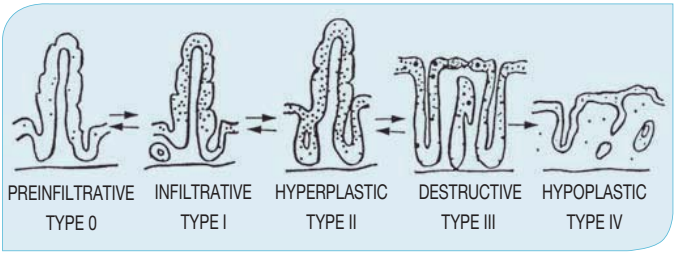


Figure 7. Aspects of mucosal pathology through the spectrum of gluten sensitivity.
(Source: MN Marsh. Proceedings of the Sixth International Symposium on Celiac Disease held at Trinity College. Dublin in July 1992).

There are two ways to obtain an intestinal biopsy sample:

- The Watson-Crosby capsule: a sample of jejunum of appropriate size is obtained from a pediatric patient under sedation.
- Endoscopy under anesthesia: multiple duodenal samples are obtained (from four to six per patient).

Not all celiac patients express the maximum degree of histological lesions before beginning a gluten-free diet. Although digestive symptoms are generally correlated to the degree of histological lesions, asymptomatic patients with villous atrophy and symptomatic patients with Marsh I histological lesions can be found, especially in the adult population [29].

Histological study of multiple samples (normally from four to six) obtained by endoscopy demonstrates the existence of patchy intestinal lesions in some cases. This possible discontinuity of the intestinal lesions may explain discrepancies between positive serology maintained over time and normal histology in a biopsy obtained by capsule, which later appears patchy by endoscopy. In daily routine practice, staining with CD3 markers for studying the percentage of intraepithelial lymphocytes is not essential.

Intestinal biopsy is recommended in patients with clinical suspicion and/or presence of positive serological markers. The absence of serological markers is not a

sufficient reason to avoid histological investigation of an intestinal biopsy (Fig. 8). In this sense, our study carried out between 1997 and 2007 shows 18 diagnosed cases of celiac disease with negative serology, 13 of them in the under-twos age group. In our experience, antibodies can be transitorily absent in children under two years of age [25].

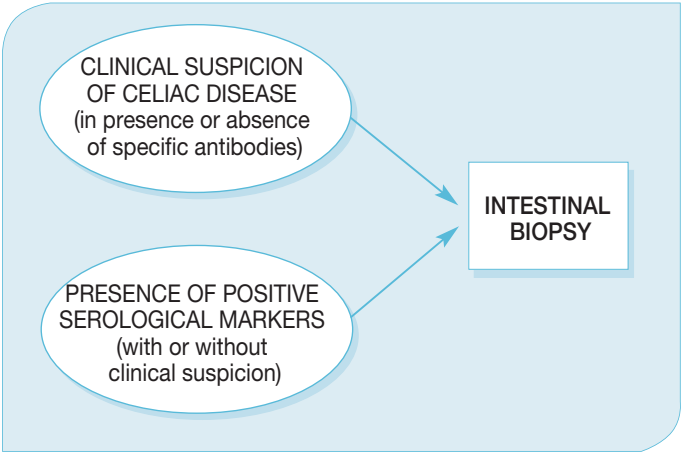


Figure 8. Histological diagnosis.

Although diagnosis is generally established through intestinal biopsy histology and appropriate response to gluten-free diet, two situations may arise for which gluten stimulation and subsequent biopsy is recommended:

- Patients on an uncontrolled diet and with negative serology.
- Patients who have begun a gluten-free diet without a previous biopsy.

In most cases, a few months on a gluten based diet causes recurrence of the initial clinical picture. However, some patients can take years to respond to gluten stimulation.

Histological lesions and response to a gluten-free diet are mostly accompanied by some of the following circumstances that support the diagnosis:

- Presence of genetic susceptibility markers HLA-DQ2 (or DQ8).
- Presence of risk factors (for example, celiac relatives).
- Presence of associated diseases (for example, thyroiditis, type-1 diabetes mellitus).

Exceptionally, histological changes associated with celiac disease may be due to other pathologies, such as tropical sprue, autoimmune enteropathy, severe malnutrition, etc.

Moreover, histological lesions can be attenuated in patients treated with immunosuppressive therapy and even when the patient is on a low gluten diet.

Markers of genetic predisposition

Celiac disease is a genetic alteration caused by the presence of DQ2 heterodimer of class II HLA (human leukocyte antigen) system.

This protein is codified for by DQA1*05 and DQB1*02 alleles and is present in one fourth of the general population.

Over 90% of patients with celiac disease are concentrated in this 20-30% of the general population that tests positive for HLA-DQ2 protein.

The few celiacs who test negative for DQ2 protein usually present DQ8 protein, codified for by DQA1*0301 and DQB1*0302 alleles.

The presence of DQ2 and DQ8 molecules is determined by analyzing the DQA and DQB alleles that codify for them, in a small sample of whole blood with EDTA. For this, genomic DNA is extracted, copies of small well-defined fragments of this DNA are produced using polymerase chain reaction with specific primers (SSP-PCR) and these copies are detected by agarose gel electrophoresis [30].

In our geographical area, 93% of celiac patients are DQ2-positive. This percentage is similar to that found in populations in northern Europe and in countries of the European Union.

In these populations, celiac patients who test negative for DQ2 protein mainly present the DQ8 heterodimer, codified for by DQA1*0301 and DQB1*0302 alleles.

Figure 9 shows that only half the celiacs who score negative for DQ2 protein in our population (2.8% of the total) present the DQ8 heterodimer, while the remaining 3.4% score negative for DQ2 and DQ8 proteins [31].

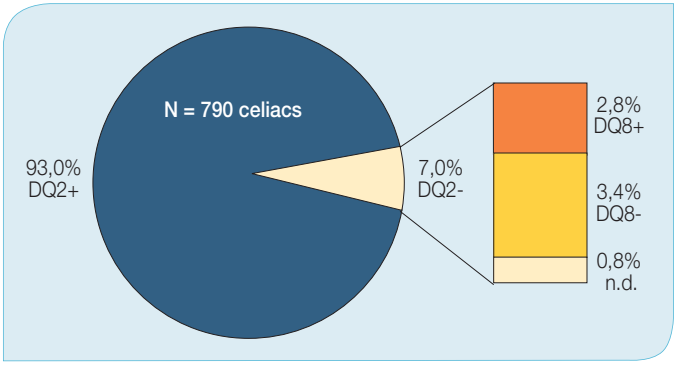


Figure 9. Markers of genetic predisposition. Our experience (1997-2007).

The usefulness of DQ2 protein in routine practice is limited, particularly when compared with serological markers or intestinal biopsy.

According to the multicenter study undertaken in Catalonia (Spain) on families of celiac patients, 64% of celiac relatives score positive for DQ2 protein and are therefore predisposed to the disease. They include 5.5% (37/675) of relatives diagnosed by serological investigation and histological confirmation. Only one in every seven relatives had clinical digestive symptoms and so could have been detected by these symptoms [24].

Of the patients with insulin-dependent diabetes (type-1) at our center, 57% score positive for DQ2 protein, and a higher risk for celiac disease is present in those who score positive for DQ2 and DQ8 proteins. They include 5-8% of the patients

affected by type-1 diabetes diagnosed with celiac disease, most of them in asymptomatic form.

Of the patients with Down syndrome in our population, 29% score positive for DQ2 protein. They include 6.3% (18/284) diagnosed with celiac disease: eight (8/18) with digestive symptoms, seven (7/18) with extra-digestive symptoms, and the remaining three asymptomatic [32].

Figure 10 compares the frequency of patients who test positive for DQ2 protein among the general population and among celiac patients, first-degree relatives, insulin-dependent diabetics and patients with Down syndrome.

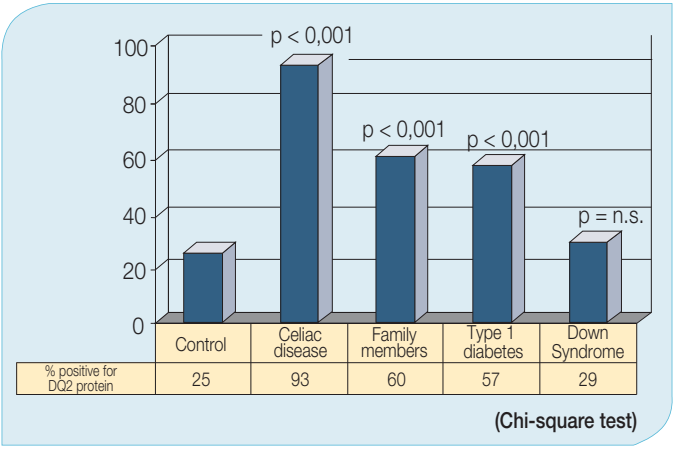


Figure 10. Frequency of DQ2 protein in at-risk populations.

The greater genetic predisposition in relatives and diabetics explains the high prevalence of celiac disease in these patients.

The higher frequency of celiac disease in patients with Down syndrome cannot be explained by greater genetic predisposition. This circumstance means better positive predictive value (PPV) for DQ2 protein in patients with Down syndrome. The positive predictive value of DQ2 protein in patients with Down syndrome is 20% (29% of DQ2-positive patients encompass 6.3% of total patients affected by celiac disease).

One in five DQ2-positive patients with Down syndrome develops celiac disease. For this reason, DQ2-positive patients with Down syndrome are susceptible to annual serological monitoring. In our experience, serological markers can appear after many years of negative results.

Figure 11 shows the results of the serological markers and genetic predisposition in the entire families of 471 pediatric celiacs in our geographical area [33]:

- The disease is excluded in 99.8% of relatives who score negative for DQ2 protein. DQ2 is therefore useful because of its negative predictive value.
- Only 8% of the relatives who score positive for DQ2 protein are found to be affected by celiac disease by serological detection and histological confirmation.

  DQ2-positive individuals with smaller lesions (Marsh I and Marsh II) can escape diagnosis if detection is serological, since specific antibodies have low sensitivity in these cases.

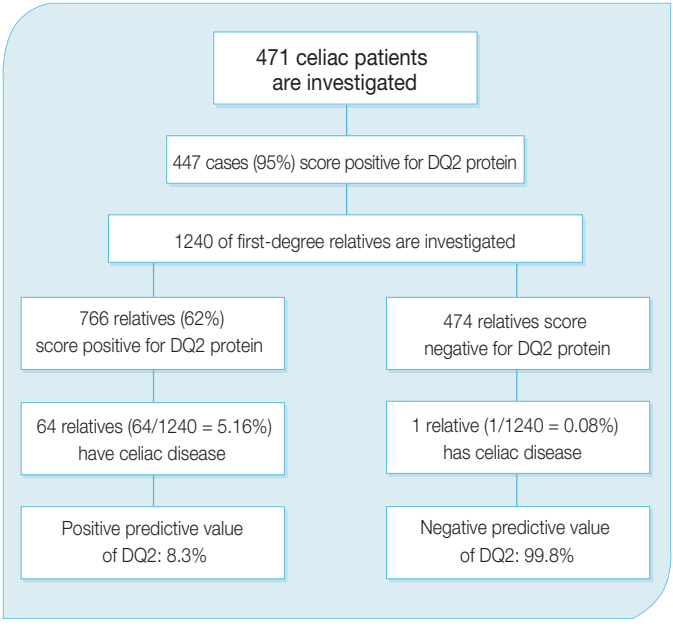


Figure 11. Study of genetic predisposition in relatives of celiacs.

Celiac disease diagnosis in first-degree relatives triples [29] (22.2% versus 7.2%) with a new strategy based on DQ2 analysis followed by intestinal biopsy versus serological detection. Patients with Marsh I and Marsh II lesions detected with this strategy showed evident clinical symptoms, mainly abdominal pain.

Finally, analysis of the alleles controlling genetic predisposition can be useful for excluding suspected

disease in epidemiological studies of the general population [34].

Regular analysis of DQ2 alleles is of interest in the following cases:

1. Patients diagnosed with celiac disease for serological and genetic investigation of their first-degree relatives.

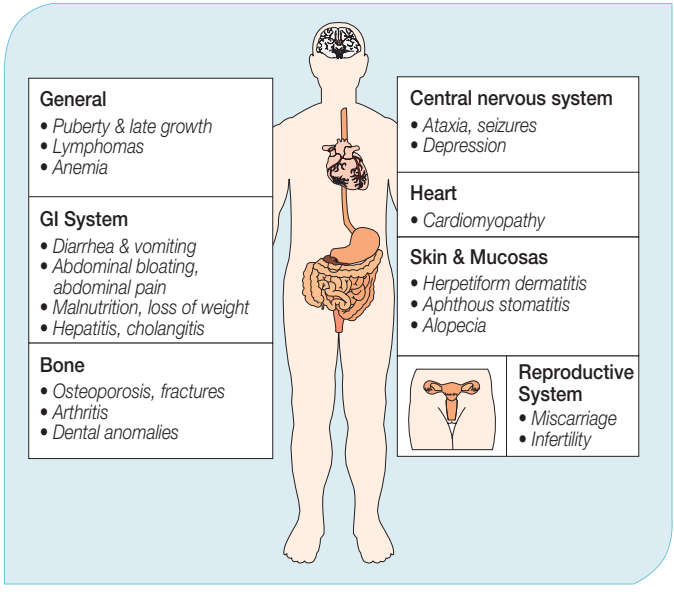


Figure 12. Celiac disease can be considered a multisystemic alteration. (Source: Rewers Marian. Epidemiology of celiac disease: what are the prevalence, incidence, and progression of celiac disease? Gastroenterology 2005; 128: s47-s51.)

2. If the DQ2 of the primary case is positive, DQ2 protein of the first-degree relatives is tested.
3. Patients suspected of having celiac disease in whom the overall clinical picture, serology and histology are not conclusive.
4. Patients from high-risk populations or with celiac disease associated diseases, in order to benefit from the negative predictive value (NPV) and to exclude the possibility of disease.

Analysis of DQ8 alleles is of interest in the following cases:

1. Patients already diagnosed with celiac disease who score negative for DQ2 protein, for serological and genetic investigation of their first-degree relatives.
2. If the DQ8 of the primary case is positive, the DQ8 protein of the first-degree relatives is tested.
3. DQ8 alleles, unlike DQ2 alleles, are not primarily related to celiac disease in the general population and, according to our criterion, their use as a risk factor for celiac disease is rather limited and should be restricted to celiac patients who score negative for DQ2 protein.

Associated pathologies

Contrary to established belief, celiac disease or *gluten-sensitive enteropathy* is a chronic disease and cannot be considered a disease exclusively of the digestive tract.

At present, celiac disease is defined as a multisystemic alteration that can be accompanied by and/or associated

with lesions in the intestine, skin, liver, joints, uterus, brain, heart and other organs in genetically susceptible persons [35].

Cutaneous and mucosal diseases
- Herpetiform dermatitis
- Alopecia
- Recurrent aphthous stomatitis
- Autoimmune urticaria

Endocrine diseases
- Type-1 diabetes
- Addison's disease
- Autoimmune thyroiditis

Osteoarticular alterations
- Osteopenia of unknown cause
- Polyarthralgia

Hepatic alterations
- Primary biliary cirrhosis
- Autoimmune hepatitis
- Autoimmune cholangitis

Neurological diseases
- Peripheral neuropathy
- Epilepsy with intracerebral calcifications
- Ataxia
- Migraine

Gynecological alterations
- Sterility
- Repeated miscarriage
- Fetal hypotrophy
- Amenorrhea

Cardiac diseases
- Idiopathic dilated cardiomyopathy
- Autoimmune myocarditis

Autoimmune diseases
- Sjögren's syndrome
- Rheumatoid arthritis
- IgA nephropathy
- Ulcerative colitis
- Crohn's disease
- Microscopic colitis

Genetic diseases
- Down syndrome
- Turner syndrome
- Isolated IgA deficiency

The clearest evidence of association with celiac disease appears in patients with herpetiform dermatitis, considered to be the cutaneous expression of celiac disease. The highest prevalence of celiac disease is found among patients with:
- Type-1 diabetes mellitus (2-5% in adults and 3-8% in children).

- Autoimmune thyroiditis (3%)
- Down syndrome (5.5%)
- Turner syndrome (6.3%)
- Isolated IgA deficiency (5.5%)

Risk populations: anemia and mild hypertransaminasemia, with no apparent cause

Iron-deficiency anemia with no apparent cause is a reason to suspect celiac disease – 2.8% to 8.7% of asymptomatic adults with anemia have celiac disease. Digestive endoscopy to investigate anemia normally includes collecting duodenal samples for histology of celiac disease. However, the frequency of celiac patients is much higher in symptomatic adults, ranging from 10.3% to 15% [21].

In pediatrics [20], one third of new celiac patients have microcytosis. Viewing this association from another perspective shows that 4% (8 out of 200) of a group of asymptomatic patients with iron-deficiency anemia with no apparent cause have celiac disease.

Serum ferritin in these 8 patients (aged from 5 to 17 years) was lower than normal in all cases, including those treated orally with iron. Three of them had associated herpetiform dermatitis, type-1 diabetes mellitus, and isolated IgA deficiency, respectively. Ferritin fell to normal levels in all cases after six months on a gluten-free diet.

Celiac disease should be considered in the differential diagnosis of iron-deficiency anemia with no apparent cause, together with *Helicobacter pylori* infection.

Mild hypertransaminasemia of unknown origin is a common alteration in patients with celiac disease and is sometimes the only indicator guiding the diagnosis.

Celiac disease can also be associated with chronic hepatic alterations such as primary biliary cirrhosis (0-6%), autoimmune hepatitis (2.9-6.4%) or sclerosing cholangitis (1.5%).

4.3% (8 out of 185) of a patient population affected by severe hepatic disease – some of whom were awaiting transplant – were diagnosed with celiac disease [36]. Of the eight cases, three were diagnosed with primary biliary cirrhosis, one with autoimmune hepatitis, and one with celiac disease.

Mild hypertransaminasemia is observed at the onset of disease in 42% of adult celiac patients and 32% in pediatric celiac patients. 9% of adults and 5% of children with mild hypertransaminasemia with no apparent cause have celiac disease [37-39]. This elevation of activity of ALT serum transaminase disappears with a gluten-free diet.

The cause of this mild hepatitis is unknown, is not related to malnutrition, and may be caused by increased intestinal permeability, permitting the hepatic spread of toxins and/or antigens through the portal vein.

In the last six years at our center, 4.4% (34 out of 775) of diagnosed celiac patients have been detected through the unexpected finding of mild hypertransaminasemia during routine monitoring.

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Notes

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