UNIVERSITÀ DEGLI STUDI DI PALERMO

FACOLTÀ DI MEDICINA E CHIRURGIA

Dottorato Internazionale di Ricerca in Scienze Stomatologiche

XXI ciclo – MED/28

Dipartimento di Scienze Stomatologiche "G. Messina"

DIAGNOSTIC POTENTIALITY OF THE ORAL MUCOSA IN PATIENTS WITH CELIAC DISEASE

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XXI ciclo – MED/28

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ABSTRACT

Celiac disease (CD) is a lifelong T cell-mediated enteropathy occurring in genetically susceptible individuals and triggered by the ingestion of gluten and related prolamins, plant storage proteins found in wheat, barley and rye which results in small intestinal mucosal injury, including villous atrophy with crypt hyperplasia and intraepithelial lymphocytosis and subsequent nutrient malabsorption. CD is characterised by a wide, and often unsuspected range of clinical presentations, including “classic” gastrointestinal symptoms (characterized by a malabsorption syndrome, ie, chronic diarrhea, abdominal pain and distention, weight loss) which are less common of the atypical CD, with few or no gastrointestinal manifestations and of the silent and latent forms.

Although the small intestinal mucosa represents the main site of the gut involved in CD, other mucosal surfaces belonging to the gastrointestinal tract and to the gut-associated lymphoid tissue can also be involved. In fact, modifications have also been found in the gastric, rectal and esophageal mucosa. Furthermore, several studies have described the determination of anti-endomysial and anti-transiglutaminase autoantibodies in media of cultured intestinal mucosa biopsies from affected patients.

A site which could be studied less invasively is the mouth, as it is the first part of the gastrointestinal system and a part of gut-associated lymphoid tissue. In fact, some oral ailments have been reported as possible atypical aspects of CD, mainly dental enamel defects and aphthous-like ulcers. Furthermore, it has been shown that inflammatory changes occur after oral supramucosal application and submucosal injection of gliadin in the oral mucosa of CD patients.

However, only few studies have focused on the histomorphological features of the oral mucosa in CD patients and until this research project was not known whether the oral mucosa of patients with CD was able to produce anti-endomysial and anti-transiglutaminase autoantibodies or not. ***The aims of the PhD study were***: a) to examine the frequency of oral lesions in adults and children with CD and to assess their usefulness in making CD diagnosis; b) to perform a screening for CD in patients with oral lesions potentially associated with CD, and sharing a common immune-mediate pathogenesis with CD, such as oral lichen planus and recurrent apthous stomatitis; c) to study the histomorphology of clinically healthy oral mucosa of untreated CD patients: (i) to assess the presence, quality (B⁄T lymphocytes) and intensity of lymphocytic infiltration and its relation to the severity of the intestinal histological damage; and (ii) to detect any other histological aspect which could be linked to CD; d) to evaluate the ability of the oral mucosa of patients with CD to produce antibodies in an *in vitro* culture system.

a) To examine the frequency of oral lesions in adults and children with CD and to assess their usefulness in making CD diagnosis. To achieve this objective two case-control studies were performed. The primary aim of the first study was to assess the frequency of oral diseases, including soft and hard tissue lesions and dental delayed eruption in CD patients, in comparison with otherwise healthy controls; the secondary objective was to consider what contribution an oral clinical examination could give to help suspect a CD diagnosis. *Patients and methods*. One hundred and ninety-seven CD patients (90 adults and 107 children) and 413 controls (180 adults and 233 children) were recruited and underwent oral examination. *Results*. Forty-six out of 197 CD patients (23%) were found to have enamel defects vs. 9% in controls (p<0.0001). Clinical delayed eruption was observed in 26% of the pediatric CD patients *vs* 7% of the controls (p<0.0001). The prevalence of oral soft tissues lesions was 42% in the CD patients and 2% in controls (p<0.0001). Aphthous-like ulcers were found in 37⁄197 (19%) CD patients *vs* 3⁄413 (1%) controls [p< 0.0001; OR=18.9505 (95% CI=9.552:37.595)]. The other soft tissue lesions detected were aspecific atrophic glossitis and geographic tongue. The first was found in 31⁄197 (16%) CD patients *vs* 1⁄413 (0.2%) controls [p< 0.0001; OR=22.464 (95% CI=10.500:48.063)], while geographic tongue was noted in 14 ⁄197 (7%) CD patients *vs* 5 out of 413 (1%) controls [p< 0.0001; OR=7.0326 (95% CI = 2.650:18.666)]. Aphthous-like ulcers disappeared in 89% of the patients after 1 year of gluten-free diet. Multi-logistic analysis selected the following variables as the most meaningful in coeliac disease patients: dental enamel defects (OR =2.652; CI =1.427–4.926) and soft tissue lesions (OR = 41.667, CI=18.868–90.909). Artificial Neural Networks methodology showed that oral soft tissue lesions have sensitivity= 42%, specificity=98% and test accuracy =83% in CD diagnosis. *Conclusions*. The overall prevalence of oral soft tissue lesions was higher in CD patients (42%) than in controls. However, the positive-predictive value of these lesions for CD diagnosis was low.

The objectives of a second case-control study were to assess the prevalence of aphthous-like ulcers in CD patients living in the Mediterranean area, and to evaluate the impact of a gluten-free diet. *Patients and methods*. A test group of 269 patients (age range 3–17 years) with CD was compared with a control group of 575 otherwise clinically healthy subjects for the presence, or a positive history of aphthous-like ulcers. CD patients with aphthous-like ulcers were re-evaluated 1-year after starting a gluten-free diet. *Results*. Aphthous-like ulcers were found significantly more frequently in CD, in 22.7% (61/269) of patients with CD *vs* 7.1% (41/575) of controls (p = < 0.0001; chi-square = 41.687; odds ratio = 4.3123; 95% confidence interval = 2.7664:6.722). Most CD patients with aphthous-like ulcers and adhering strictly to gluten-free diet (71.7%; 33/46) reported significant improvement on gluten-free diet, with no or reduced episodes of aphthous-like ulcers (p = 0.0003; chi-square = 13.101; odds ratio = 24.67; 95% confidence interval = 2.63:231.441).

Conclusions. The epidemiological association found between CD and aphthous-like ulcers suggests that recurrent aphthous-like ulcers should be considered a risk indicator for CD, and that gluten-free diet leads to ulcer amelioration.

b) To perform a screening for CD in patients with oral lesions potentially associated with CD and sharing a common immune-mediate pathogenesis with CD. In a first study the assessment of the prevalence of CD in patients with oral lichen planus and the effects of the gluten-free diet on this oral disease was performed. *Patients and methods*. Twenty-three patients with oral lichen planus confirmed both clinically and histologically were tested for serum coeliac autoantibodies. All patients with positivity to anti-endomysial and/or anti-transiglutaminase autoantibodies underwent small intestinal biopsy to confirm CD, and in case of confirmed diagnosis were re-evaluated after 3 months of gluten-free diet. *Results*. Only 2/23 (8.7%) female patients with atrophic/erosive oral lichen planus localized on buccal mucosa, associated to burning sensation, despite topical corticosteroid therapy, showed positive values of celiac antibodies associated to low serum levels of folate, vitamin B12 and iron. The small intestinal biopsy confirmed the diagnosis of CD. A gluten-free diet was started and the oral soreness and anaemia gradually improved over the next 3 month. *Conclusions*. It is uncertain whether the oral lesions are a direct manifestation of gluten enteropathy or due to the effect of malabsorption and of the consequently haematinic deficiencies on the rapidly dividing mucosal cells already predisposed to soreness by pre-existing lichen planus. However, the resolution of the burning sensation after gluten-free diet, in patients with oral lichen planus and refractory to local therapy could be justify the second hypothesis. Furthermore, a search for underlying nutritional deficiencies in patients with atrophic/erosive oral lesions should be important in relieving oral soreness.

In a second study the assessment of the prevalence of CD in patients with recurrent aphthous stomatitis was performed. *Patients and methods*. Twenty-five patients with recurrent aphthous stomatitis were included. The patients had at least three ulcerative episodes per years. Patients were screened by IgA anti-endomysial antibody, IgA anti-tissue transglutaminase and serum IgA level. Those with a positive serology underwent endoscopic biopsies of the duodenal mucosa. *Results*. Two out of 25 recurrent aphthous stomatitis patients had a positive CD serology and the histological findings were compatible with gluten-sensitive enteropathy. *Conclusions*. Although our study concerned a limited sample population of patients with recurrent aphthosis, it showed that a good percentage (8%) of patients with recurrent aphthous stomatitis had CD. If this results is compared with the 1% prevalence of CD in general population of Italy this study suggest that evaluation of CD could be appropriate in patients with recurrent aphthous stomatitis.

c) To study the histomorphology of clinically healthy oral mucosa of untreated CD patients in order: (i) to assess the presence, quality (B/T lymphocytes) and intensity

of lymphocytic infiltration and its relation to the severity of the intestinal histological damage; and (ii) to detect any other histological aspect which could be linked to CD. *Patients and methods*. Twenty-one untreated CD patients (age range 13–68 years) with clinically healthy oral mucosa were enrolled and compared with 14 controls. Intestinal and oral biopsies were carried out and specimens were evaluated after staining with hematoxylin and eosin. *Results*. Intra-epithelial lymphocyte B and T infiltrates of the oral mucosa were found to be similar in both groups; likewise, intensity of the lymphocytic infiltrate in the lamina propria was similar in both groups and was not related to intestinal damage; important signs of spongiosis of the oral mucosa epithelium were found to be more significantly present in CD patients (90%) compared with controls (p=0.0002). This spongiotic reaction was characterized by the presence of intraepidermal and intercellular oedema; intercellular spaces were widened with an elongation of the intercellular bridges. The foci of spongiosis were also characterized by a mild lymphocytic infiltrate, but not by the presence of eosinophils or neutrophils. These lesions were exclusively microscopic without any corresponding identifiable vesicles on the oral mucosal surface. *Conclusions*. This study showed that the healthy oral mucosa of untreated patients does not reflect the intestinal damage by CD, but it is unexpectedly affected by spongiosis, as being detected for the first time in the literature. This latter feature could be related to gliadin ingestion and could contribute to explain the higher susceptibility of CD patients to suffering from oral mucosa lesions.

d) to evaluate the ability of the oral mucosa of patients with CD to produce antibodies in an in vitro culture system. *Patients and methods*. Twenty-eight patients with new diagnosis of CD (15 adults and 13 children) and 14 adult subjects with other diseases (controls) were studied. All underwent oral mucosa biopsy and subsequent anti-endomysial and anti-tissue transglutaminase assays on the mucosa culture medium. *Results*. Sensitivity and specificity of anti-endomysial and anti-tissue transglutaminase assayed in the oral mucosa culture medium for CD diagnosis were 54% and 100% and 57% and 100%, respectively. The diagnostic accuracy of anti-endomysial and anti-tissue transglutaminase assayed in the oral mucosa culture medium for CD diagnosis was 79%. The CD clinical presentation, such as the presence of oral mucosa lesions, did not influence the results of the anti-endomysial and anti-tissue tranglutaminase assays in the oral mucosa culture medium. There was an association between positivity of antibodies and greater severity of the oral mucosa lymphocyte infiltration. *Conclusion*. This study demonstrates for the first time that the oral mucosa contributes to anti-endomysial and anti-tissue transglutaminase production in untreated patients with CD.

In summary, the researches conducted in the context of this project, have yielded important results not only in terms of prevalence of oral lesions potentially associated with CD, but

above all, thanks to the highly innovative research lines has led to consider the oral cavity, for the possibility to perform easily oral biopsies to be processed with appropriate methods of immunofluorescence and conventional histological examination, as a potential diagnostic gate minimally invasive and easily accessible in comparison with the endoscopic approach. However, clinical trials conducted on largest sample size and on CD patients before and after the gluten-free diet, will need to see if the mouth can really be helpful to duodenal endoscopy for the first diagnosis of CD, but also in the follow-up of the disease. Furthermore, the demonstration that the oral mucosa is a new site involved in the release of pathognomonic autoantibodies of CD and which may show some histological peculiarities offers us a site to obtaine bioptic samples to be underwent cDNA microarrays studies and cell and tissue cultures, in order to depth the pathogenesis of CD and to test less toxic compounds in the treatment of this condition.

LIST OF ORIGINAL PRODUCTS

This thesis is based on the results of the studies performed by Dr. Domenico Compilato about the research project entitled "*Diagnostic potentiality of the oral mucosa in patients with celiac disease*" and conducted during the three years of the *PhD* course. These results have been object of the following original publications on national and international scientific journals and/or communications at national and international conferences.

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ABBREVIATIONS

CD	celiac disease
GSE	gluten sensitive enteropathy
HLA	histocompatibility leukocyte antigen
IEL(s)	intraepithelial lymphocyte(s)
tTG	tissue-transglutaminase
EMA	anti-endomysial antibody
AGA	anti-gliadin antibody
DEDs	dental enamel defects
RAS	recurrent aphthous stomatitis
ALU	aphthous-like ulcers
GFD	gluten-free diet
GT	gastrointestinal tract
GALT	gut-associated lymphoid tissue
NALT	nasal-associated lymphoid tissue
MALT	mucosal-associated lymphoid tissue
sIgA	secretory IgA
OLP	oral lichen planus
MHC	major histocompatibility complex
DH	dermatitis herpetiformis
GVHD	graft versus host disease
TJ	tight junctions
SNP(s)	single nucleotide polymorphism(s)
SS	Sjögren’s syndrome
OR	Odds ratio
CI	Confidence Interval
ANN(s)	Artificial Neural Network(s)

ROC	Receiver Operating Characteristic
PBS	phosphate-buffered saline
CV	coefficient of variation
EGDS	esophago- gastro-duodenoscopy
H&E	hematoxylin and eosin
OD	optical density

INTRODUCTION

It was until 1987 that attention was drawn to celiac disease (CD), when Dr. Samuel Gee described the classical features of the disease in children[1] as diarrhoea, lassitude, and failure to thrive. At that time he realised that the diet was important in the treatment and noted that the diet was not age-specific[1,2]. In 1953, the Dutch paediatrician Dicke presented his thesis on the role of gluten in CD patients[3]. He demonstrated in a controlled study, that wheat, rye and barley triggered CD, and that the condition could be reversed after their exclusion from the diet[3].

Today it is known that CD, also known as celiac sprue and gluten sensitive enteropathy (GSE) is an immune-mediated enteropathy triggered by the ingestion of gluten, the storage protein of wheat, in genetically susceptible individuals[4]. In this context, the term “gluten” is used to refer to prolamins of wheat, rye (secalins) and barley (hordeins)[5]. There is scientific evidence that avenins of oats are less harmful than glutens[6,7]. The major predisposing genes are the histocompatibility leukocyte antigen (HLA)-DQ2 and DQ8 genotypes found in at least 95% of patients[5], although non-HLA genes have been recently reported to be involved in CD pathogenesis[8].

CD, originally through to occur only rarely in childhood, is now recognised as a common condition that could be diagnosed at any age[5].

Until recently considered a rare condition, CD is now well-known to be relatively common; its estimated prevalence in the general population of North America and Western Europe appears to be close to 1%, with a reasonable range of 0.71% to 1.25%[9]. CD prevalence is increased in at-risk conditions such as family history of CD, autoimmune diseases, especially type 1 diabetes and thyroiditis, IgA deficiency, and some genetic syndromes (Down, Turner, and William syndromes)[10].

CD affects primarily the small intestine; in fact, in individual with CD, gluten induces typical small intestinal histological damage with villous atrophy or flat duodenal and jejunal mucosa, crypt hyperplasia, intraepithelial lymphocyte (IEL) accumulation, and numerical increase of plasma cells in the lamina propria. These histopathological changes lead to subsequent nutrient malabsorption and clinical manifestations[11].

The development of GSE is paralleled by the appearance of serum antibodies, especially the IgA class anti-tissue transglutaminase (anti-tTG), anti-endomysial antibody (EMA) and antigliadin antibodies (AGA), and eventually clinical manifestations.

The dramatic advances made during the last decade in the understanding of CD have revealed the disease to have a heterogeneous, wide, and often unsuspected range of clinical presentations. In fact, today it is apparent that the ‘‘classic’’ clinical form (characterized by a malabsorption syndrome, i.e., chronic diarrhea, abdominal pain and

distention, weight loss) is less common, and most patients have atypical CD, with few or no gastrointestinal symptoms and predominance of extraintestinal manifestations, such as short stature, iron-deficient anemia, abnormalities in liver function test[12]. Furthermore, silent and latent forms have also been described[5]. CD patients are prone to develop long-term complications (eg, osteoporosis, infertility, autoimmune diseases, malignancies)[13], and some studies report an increased all-cause mortality compared with the general population[14,15]. The wide range of the CD clinical manifestations reluts in patients presenting to many different specialists: gasteoenterologists, endocrinologists, rheumatologists, haematologists, cardiologists, neurologists, as well as paediatricians, dermatologists and dentists to consider this disorder when a patient presents with those extraintestinal signs and symptoms that might be related to CD[5]. In this regard, also some oral ailments have been reported as possible atypical aspects of CD, mainly dental enamel defects (DEDs) and recurrent aphthous stomatitis (RAS) or better aphthous-like ulcers (ALU)[16,17].

In active CD, the serum of patients contains autoantibodies against self-antigens including endomysium and tTG[18,19]. In many cases the presence of EMA and anti-tTG antibodies, and their disappearance after a gluten-free diet (GFD), confirms the diagnosis[20]. Despite the high sensitivity and sensibility of the today available CD serological tests, in many cases the diagnosis should not be based on symptoms of the patients or findings in the serological testing alone[7] and small intestinal biopsy sampling is the gold standard. Furthermore, recent avidence shows that the diagnostic accuracy of an intestinal biopsy can be greatly increased by measung ant-tTG and EMA produced *in loco* by duodenal mucosa [21-24].

Although novel therapies have been proposed[8], the strict and lifelong adherence to a GFD is the only treatment currently available, and usually results in remission. Compelling evidence suggests that GFD may prevent or reduce the risk of long-term complications.

Although the small intestinal mucosa represents the main site of the gut involved in CD, other mucosal surfaces belonging to the gastrointestinal tract (GT) and to the gut-associated lymphoid tissue (GALT) can also be involved. In fact, modifications have also been found in the gastric [25,26], terminal ileal[27] and rectal mucosa[28-33]. Rectal mucosa, furthermore, has also been used for gluten challenges[32,34-38]. Finally, esophageal involvement has also been shown in patients with untreated CD[25,39]. All this demonstrates that gluten-driven T-cell activation is not restricted to the proximal part of the intestine but is present in the whole intestine.

A site which could be studied less invasively is the mouth; in fact the oral cavity mirrors well our general being. The oral cavity is closely bound to the immunogenic defence systems. The lymphoepithelial structures of Waldeyer's pharyngeal ring in the posterior part of the oral cavity, including oral mucosal and nasal-associated lymphoid tissues (NALT) constitute an important part of GALT. GALT is the major part of mucosal-associated lymphoid tissue (MALT)[40]. After antigen-activation, proliferation and partial differentiation in MALT, memory

B and T cells migrate to regional lymph nodes and secretory glands, including salivary glands where they can begin to produce secretory immunoglobulin class-A (sIgA).

As the first part of GT, the oral cavity is the route for all materials ingested, including products made from cereals. It might be suspected that oral cavity would react to ingested gluten in the same way as the mucosa of the gut, showing oral changes when the disease is untreated and improvement in these changes when CD is treated with GFD.

In this context, Lahteenoja *et al*. showed that local challenge of the oral mucosa in treated CD patients provoked a gluten-dependent mucosal immune response, displayed by the increase of intraepithelial CD4+ T cells, as well as of CD4+ and CD8+ T cells in the *lamina propria*[41]. This feature suggest that, in CD patients, the mucosal surface of the mouth could respond to gluten in a similar way to the mucosa of the small intestine. Nevertheless, the same group later failed to find significant immunological differences in oral mucosa between untreated CD and control patients[42]. In some recent studies, detection of salivary anti-tTG in CD patients re-opened the possibility that the mouth might be actively involved and used as a new site for detection of CD[43-45].

However, only few studies have focused on the histomorphological features of the oral mucosa in CD patients and until this research project was not known whether the oral mucosa of patients with CD was able to produce EMA and anti-tTg autoantibodies or not.

In this light, ***the aims of this PhD study were***: a) to examine the frequency of oral lesions in adults and children with CD and to assess their usefulness in making CD diagnosis; b) to perform a screening for CD in patients with oral lesions potentially associated with CD, and sharing a common immune-mediate pathogenesis with CD, such as oral lichen planus (OLP) and recurrent apthous stomatitis (RAS); c) to study the histomorphology of clinically healthy oral mucosa of untreated CD patients: (i) to assess the presence, quality (B/T lymphocytes) and intensity of lymphocytic infiltration and its relation to the severity of the intestinal histological damage; and (ii) to detect any other histological aspect which could be linked to CD; d) to evaluate the ability of the oral mucosa of patients with CD to produce antibodies in an *in vitro* culture system.

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CHAPTER I

Review of the literature

1. Celiac disease

1.1. Historical background

CD was first described in the second century AD by Aretaeus of Cappadocia[1], a contemporary of the Roman physician Galen, who used the Greek word "*koeliakos*", which means "*suffering of the bowels*". He described the characteristic stools and chronicity of the disease, noting it to be present also in children. Vincent Ketelaer, a Duch physician, for the first time described the oral aphthous ulceration associated with sprue as early as 1669[2]. However, only in 1888 AD did Samuel Gee of St. Bartholomew's Hospital give the classical clinical description of CD in his classical paper: On the Coeliac Affection. He described the disease in children. Dr. Gee's description of the features of the disease was remarkably accurate and he even suggested that the disease might be treated by means of a diet[3]:

> *"There is a kind of chronic indigestion which is met in persons of all age, yet is especially observed in children between one and five years."*

Gee supposed that the diet is important:

> *"The causes of the disease are obscure. Children who suffer from it are not all weak in constitution. Errors in diet may perhaps be a cause, but what error? Why, out of a family of children all brought up in much the same way, shoul on alone suffer? This often happens…To regulate the food is the main part of the treatment…..Malted food is better, also rusks or bread cut thin and well toasted on both sides….The disease being a failure to digestion, nothing seems more reasonable, at first sign, than to digest the patient food artificially before it is given….But if the patient can be cured at alla, it must be by means of diet."*

The natural course of CD was very often very miserable before the understanding of the importance of gluten in diet:

> *"Naked-eye examination of dead bodies throws no light upon the nature of the coeliac affection: nothing unnatural can be seen in the stomach, intestines, or other digestive organs. Whether atrophy of the glandular crypts of the intestines be ever or always present, I cannot tell".*

Many theories on CD have been introduced since Gee's paper.

In the 1920s, Haas introduced a banana diet excluding bread, potatoes and cereals from the diet as treatment of CD[4]. This might account for part of the success of this banana diet. Blumgart, in 1923, on post-mortem histological examination of a small bowel wall, detected a thin atrophic mucosa with shortened villi and lymphoid cell infiltration[5]. However, the investigators attributed generally morbid anatomical findings in the small intestine of CD patients to post-mortem changes[6]. These views dominated the thinking on the subject until 1950s. Still more observations were made on atrophy of the small intestinal mucosa with morphological changes in the villi and chronic inflammatory cell infiltration in the lamina propria[7-9].

No real progress in treating the disease was made until the 1930s-1950s, when WK Dicke, a Dutch pediatrician, showed that the health of celiac children dramatically improved when wheat, rye and barley, which were unavailable during the 2nd World War, were removed from their staple diet, only to relapse at the end of the war when the consumption of wheat flour started afresh in the Netherlands[10].

The dietary treatment of CD has remained unchanged until today, but its discovery stimulated an enormous amount of research to find out the mechanisms by which gluten brings about malabsorption.

Finally, the stage was set for the introduction of another important advance in the understanding of CD – the technique of jejunal biopsy. At the same time, Shiner in England and Royer and co-workers in Argentine were independently able to obtain biopsy specimens of the jejum as well as the stomach[11,12]. Since the introduction of per-oral intestinal biopsy instruments, the autopsy findings and per-oral biopsy findings could be correlated, and it was detected that the major site of CD lesion is the small intestine[9]. This procedure has become the diagnostic standard in the avaluation of patients with suspected CD[13].

CD is the result of both environmental (gluten) and genetic factors (HLA and non-HLA genes), and the distribution of these two components can probably be used to identify the areas of the world at risk for gluten intolerance. In this respect, the world geographical distribution of CD seems to have followed the spread of wheat consumption and the migratory flows of mankind. Indeed, man was not originally a gluten eater, but led a nomadic life obtaining food by hunting, fishing and collecting fruit as well as vegetables, and for hundreds of thousands of years never had any contact with gluten-containing cereals. Only about 10000 years ago in a small region of South Western Asia, called the “Fertile Crescent” including Anatolia (Southern Turkey), Lebanon, Syria, Palestine and Iraq, were wild grains (wheat or *Triticum Dicoccoides* and barley or *Hordeum Spontaneum*) cultivated, due to the special environmental conditions created by the flooding of the Tigris and Euphrates. In the Fertile Crescent some tribes changed from a nomadic lifestyle to one of stable settlement because land cultivation permitted food storage, and they later migrated

westwards because new lands for cultivation were needed. They spread through the Mediterranean area (Northern Africa, Southern Europe) and the Danube valley (Central Europe) and their expansion continued from 9000 to 4000 BC by which time the cultivation of wheat and barley had spread all over the Old Continent, also reaching Northern Europe (Ireland, Denmark and the Scandinavian countries). However, this expansion in farming was not limited to the diffusion of agricultural practices, but was also a “demic” expansion, because the peoples coming from South West Asia replaced the local inhabitants. Hence, the European and North-African populations share a genetic background with the peoples of South West Asian origin (Middle-East), including also DR3-DQ2 and DR4-DQ8, the CD-predisposing haplotypes[14].

1.2. Epidemiology

CD is more common in female than in male with a female to male ratio generally accepted to be 2-2.1:1, although some have suggested it my be more equal[15].

CD, originally through to occur only rarely in childhood, is now recognised as a common condition that could be diagnosed at any age.

On the basis of the clinical frequency, CD was previously regarde as rare, occurring in 1 in 3345 people worldwide[16]. However, serologic screening studies have shown the worldwide prevalence to be 1 in 266[17]. Such a rate established CD as one of the most common genetically based disease[18].

The epidemiological changes of CD are efficiently conceptualized by the "iceberg model", originally introduced by Richard Logan in 1991[19]. In fact, until the introduction of the more accurate serological diagnostic CD test, the atipycal, silent or latent forms remained clinically undetected. The prevalence of CD can be conceived as the overall size of the iceberg, which is primarily influenced by the frequency of the predisposing genotypes in the population.

In fact, the most important determinants of prevalence in any population are the frequency of major histocompatibility complex (MHC) class II alleles that encode for HLA-DQ and exposure to dietary products that contain gluten. HLA-DQ is a cell surface dimer, largely restricted to antigen-presenting cells, that presents foreign peptides to T lymphocytes. The DQ dimer is composed of non-covalently associated alpha and beta chains encoded by *HLADQA1* and *HLA-DQB1* genes, respectively. As *DQA1* and *DQB1* genes are inherited from each parent, each individual has two *DQA1* and two *DQB1* genes[20]. These can be identified serologically as DQ types 1–9 or genetically as over 100 separate alleles. Genetic characterization is now widely used, although some laboratories continue to use both genetic and serological typing. The contribution of HLA type to the genetic risk for CD has been variously estimated at 36–53%[21,22]. Approximately 90% of patients are serotyped as DQ2, whereas most of the remainder have DQ8. With genetic testing, DQ2 is almost synonymous with *DQB1*02*, a gene with two common alleles designated *DQB1*0201* and *DQB1*0202*. Another consideration is strong linkage disequilibrium between *DQB1*0201*, *DQA1*0501* and *DRB1*03* creating the serologically defined haplotype, DR3-DQ2.5. The frequency of DQ2 in Caucasian populations in western Europe has been estimated at 20–30% and relatively high frequencies also occur in northern and western Africa, the Middle East and central Asia. Thereafter, the frequency of DQ2 declines from west to east with low frequencies in populations in South-East Asia and the virtual absence of DQ2 in Japan. Overall, only one of approximately 30 people with DQ2 will develop CD, although much more complex analyses of risk have been associated with HLA antigens that have been defined by genotyping[22].

The second important determinant of CD is exposure to gluten-containing cereals, representing also the primary environmental factor. So it is logical to assume that the geographical distribution of CD reproduces, at least in part, that of gluten-containing cereals consumption. Domestication of ancient grasses began in the "*Fertile Crescent*" of western Asia approximately 8000 years ago and subsequently spread to Africa, India and western Europe. Globally, wheat is the most consumed cereals in the human diet (according to FAO, availability per capita in 1998 amounted to 71.5 kg per year compared to 58.1 kg/year of rice).

In Developing Countries, the availability of wheat (63.4 kg/year) is second to that of rice (71.4 kg/year), but is increasing more rapidly than the latter (in 1961s was 54, 4 kg/year of rice and 29.3 kg/ year of wheat). This trend could lead to celiac "pandemy", related, probably, on the progressive "Westernization of food worldwide".

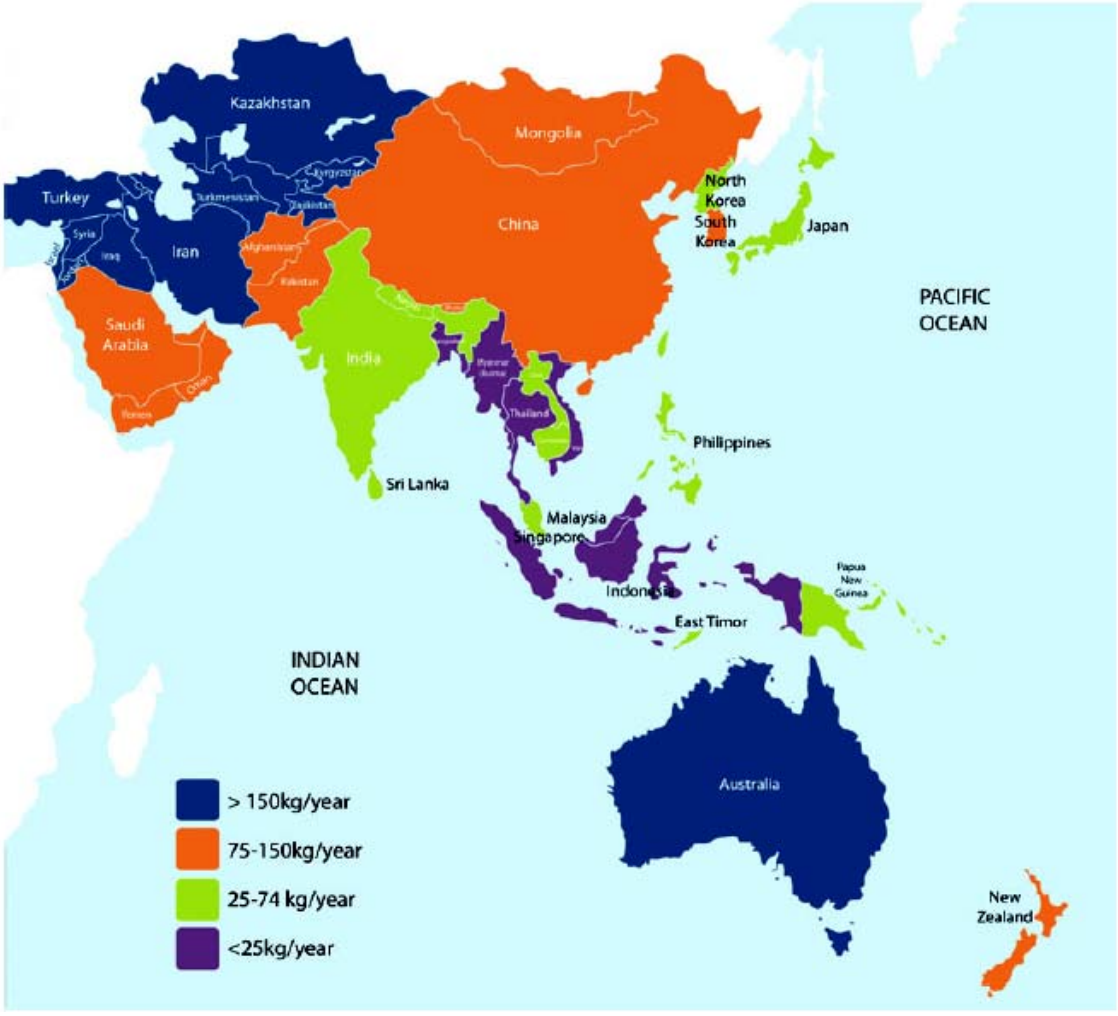


Figure 1. *Wheat consumption (Kg) per person per year for countries in the Asia-Pacific region*[20].

In fact, although wheat is the staple cereal of most Caucasian populations, the diet of many populations in Asia and South-East Asia is based on rice. In some countries such as India, diets vary in different regions with wheat cereal as the staple diet in north-western states

and rice as the staple diet in most of the southern states. An additional issue is the increasing use of wheat-based products associated with urbanization and rising incomes in areas of Asia that were once considered traditional rice-eating regions. The current wheat consumption per capita per year in countries in the Asia–Pacific region is shown in *Figure 1*. Until a few years ago, gluten intolerance was thought to be a disorder almost exclusively affecting Europeans or people of European origin (North Americans and Caucasian Australians) and the phenotype blue eyes and blond hair was described as typical of CD patients[14,23]. In this respect, serological screening in the general, unselected populations of the Western world, North America and Australia clearly demonstrated that the prevalence of gluten intolerance in these areas of the world likely ranges from 0.5%-1%, that is from 1:200 to 1:100 (Table I).

Table I. *Prevalence of CD in Europeans and people of European ancestry based on unselected population serological screening.*

Europe	
Czechoslovakia	1:218
Estonia	1:88
Finland	1:99
Hungary	1:85
Ireland	1:122
Italy	1:106
Norway	1:262
Portugal	1:134
Spain	1:118
Sweden	1:190
Switzerland	1:132
Netherlands	1:198
United Kingdom	1: 100
United States	1:133
Australia	1:251

On the other hand, until recent years CD had been observed only in sporadic cases among native African immigrants in Europe[14,24-26], in a few African-Americans serologically screened for CD in the United States[14,16,27], and in one black patient of South Africa[28]. Similarly, until a few years ago, there were only limited case studies and occasional observations of CD in Latin America[14,29-32], in North Africa[33,34]; and in the Middle East[35-37], where gluten intolerance was believed to be rare. In addition, CD has been historically[17] considered absent in the Far East (China, Japan, Korea, Malaysia, etc.).

In contrast, recent large screening studies performed by means of simple, sensitive and specific tests (AGA, EMA and anti-tTG antibodies assays) on the general population and at-risk groups in those developing areas of the world where there is a large consumption of wheat, such as North Africa, India and Middle East, showed that the prevalence of gluten intolerance had been underestimated and that it is, instead, similar to that of the so-called Western countries.

In African populations, specifically in the Maghreb area (the Northern Region of Africa including Morocco, Algeria, Tunisia, Libya and Egypt) very high incidences of CD have recently been reported both in the general population[38,39] and in at risk-groups.

Serological screening in 2500 Tunisian healthy blood donors showed that the prevalence of EMA in the general population is 1:355, which is close to that of Europeans[40]. These high frequencies are not surprising because wheat and barley are the major staple foods in the Maghreb countries[40], and because there is a high frequency of the DR3-DQ2 CD predisposing haplotypes in these populations[41,42].

Another population in North Africa with an elevated prevalence of CD (5.6%), which is the highest known in the world today[42], is the Saharawi people, who are of Arab and Berber origin, who have a high degree of consanguinity, and who live as refugees in Algeria (Sahara Desert). This elevated prevalence may be explained both by genetic factors, as the Saharawi population has a very high frequency of the DR3-DQ2 haplotype, and by environmental factors, because in the last few decades they have changed their dietary habits. For example, the rates and duration of breast-feeding have been reduced and large amounts of gluten are now being consumed in early life as part of the staple diet, due to the humanitarian aids supplied by Western countries[43]. However, other genetic and environmental factors probably play an important role in explaining such a high frequency of CD in the Saharawi people, because gluten-containing foods are also the staple diet in Sardinia and similar frequencies of DR3-DQ2 have been observed in the Sardinian population, but here there is a much lower prevalence of CD[44].

CD is a relatively common cause of chronic diarrhea in Iran, Iraq and Kuwait and has been diagnosed in 2–8% of patients with type 1 diabetes in Iran, Israel and Saudi Arabia[45]. Many of these countries have a per capita wheat consumption that ranks among the highest in the world. Although only a limited number of genetic studies have been carried out, the population of countries such as Iran, Saudi Arabia and Turkey have a high frequency of *HLA-DQB1*02* . Using adult blood donors, prevalence rates for celiac disease in Iran, Israel, Syria and Turkey were 1:166 (0.6%)[46], 1:157 (0.6%)[47], 1:62 (1.6%)[14] and 1:87 (1.2%)[48], respectively. Similar prevalence rates were determined in surveys of Iranian children (1:165, 0.6%)[49] and Turkish children (1:115, 0.9%). These prevalence rates are almost identical to those of a variety of countries in Europe[23].

These data on the new epidemiology of CD among Middle Eastern people do not appear surprising because these populations live in countries included in the "*Fertile Crescent*", the region where CD originated and where there is both a large consumption of wheat [45,50] and a high frequency of the HLA CD predisposing genes[51,52].

In Southern Asia there are still no data available on CD prevalence in the general and unselected populations. Therefore, because in past decades reports of gluten intolerance were sporadic, CD was believed to be very rare in this area of the world[53-55]. In contrast, several recent studies suggest that gluten intolerance is also common in South Asia. For example, CD has been diagnosed in 26% to 49% of Indian children presenting with chronic diarrhea at tertiary care hospitals[56,57], and during the last few years a large number of CD patients have been observed in many case studies in the Indian Sub-continent, especially in Northern India[58-61]. In addition, during these years some studies[62] reported CD in South Asian patients who had immigrated to Europe or North America. In this respect, a recent Italian multi-center study on immigrant children with CD showed that 3 were native to Pakistan and 1 to Sri-Lanka (formerly Ceylon, to the south of India), while in previous studies[63,64] respectively 10 and 13 CD children of Punjabi descent were reported in the UK. Interestingly, in the Punjab (Northern India), gluten intolerance is called "*summer diarrhea*" and in summer the "*chapattis*", which are the typical staple food, are made of wheat while in the winter maize flour is used. However, the people living in South Asia have the CD-predisposing HLA genes because they are Aryan in origin[65-67] and their staple diet is rich in wheat-derived foodstuffs. These data clearly support the hypothesis that CD is widespread in South Asia, but likely under-diagnosed. Therefore, both greater attention and awareness among physicians as well as mass serological screenings in the general populations are needed to establish the real prevalence of CD in these countries[68].

CD has been historically[17,69] considered absent in the Far East (China, Japan, Korea, Malaysia, etc.). On the contrary, recently three cases of gluten intolerance have been observed in Canada among adult descendents of Japanese (two patients) and Chinese (one patient) immigrants[63]. This finding, even when limited suggests that genetic susceptibility to CD exists also among these populations. It might be related to genes different from DR3-DQ2 and DR4-DQ8 haplotypes, as these are rare or absent in these areas of the world[17,70]. Furthermore, HLA-DQB1*02 is virtually absent from the Japanese population but is present at low frequency in the Chinese population. However, other questions arise in this regard.

CD is likely to be rare in Indonesia, Korea, the Philippines and many smaller Pacific islands because of low wheat consumption and a low frequency of HLA-DQB1*02. In South-East Asia, HLA-DQB1*02 is often present in more than 5% of the population but CD is predicted to be rare, as staple diets are based on rice. In contrast, prevalence rates that are similar to those in Europe are likely to apply to most of the Middle East and may also apply to

countries that extend from Pakistan in the south to Kazakhstan in the north. Ancient migration patterns that determine the frequency of DQB1*02 would also predict more patients with CD in western China than in eastern China. *Figure 2* shows the prevalence of CD in Asia-Pacific Region[20].

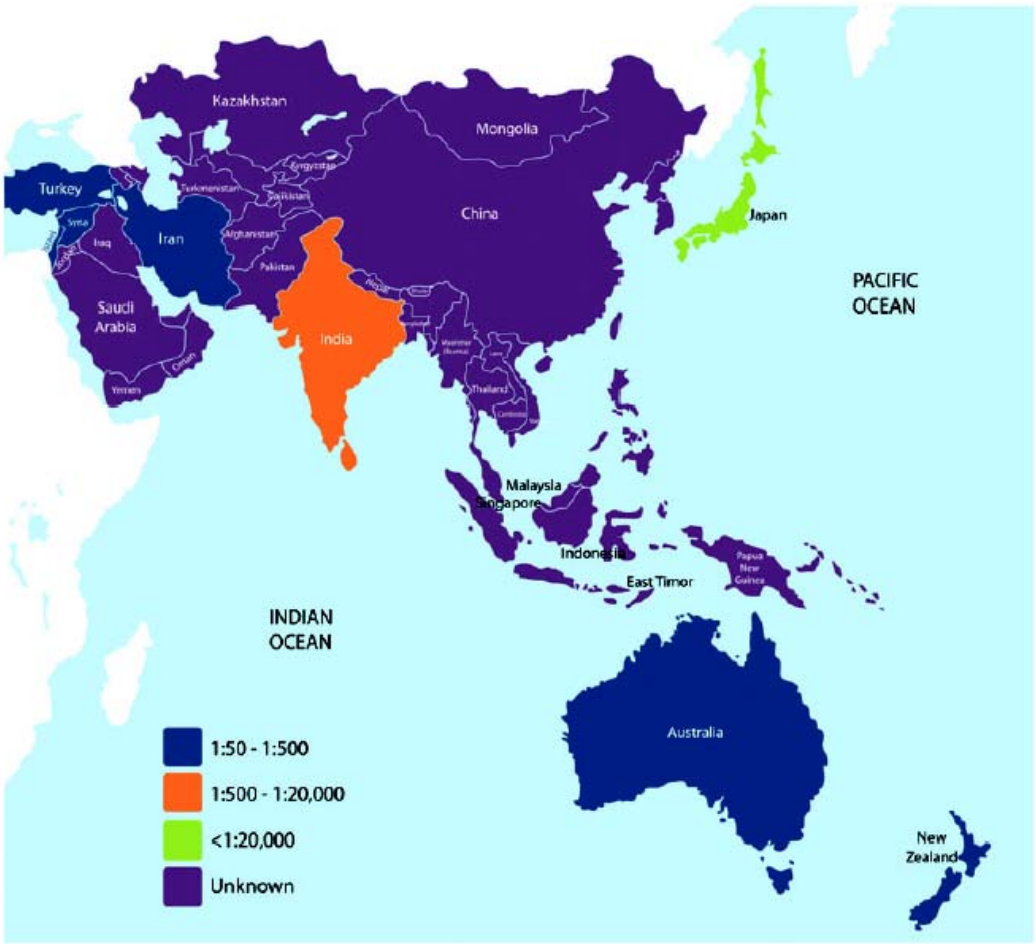


Figure 2. *Prevalence of celiac disease in countries in the Asia–Pacific region. For the majority of countries, the prevalence is unknown[20].*

CD is a common disorder in Latin America, both in the more developed (e.g. Brazil and Argentina) and in the less developed (e.g. Cuba, Chile, Uruguay) countries. This phenomenon is noteworthy because a large proportion of Latin American people share common European ancestry and because wheat is commonly present in their staple diet. A high prevalence (1:167; 0.60%) of undiagnosed CD cases has been recently observed following serological screening among the general population in Argentina[71], in Uruguayan children (1:51; 1.96%) and in 2 studieson *healthy* Brazilian blood donors, 1:276 (0.36%) and 1:292 (0.34%) respectively[14]. These rates are higher than those of the first serological screening performed on a general population in Latin America (i.e. Brazil), where the observed prevalence of undiagnosed subclinical CD patients was 1:681 (0.15%). However, this lower prevalence might be due to the fact that most of the studied subjects were men

(while CD is more frequent in women), that anemic subjects were excluded from the screening in the study design (iron-deficiency anemia is a very common atypical form of CD), and that a single serological test (IgG AGA assay) was used as a first-level screening test[14].

In Latin America there is also a high prevalence of gluten intolerance among the groups at risk. In Cuba, undiagnosed CD prevalence among children with type-1 diabetes mellitus, with Down's syndromeand among undernourished children was respectively 2.5%, 2.3% and 2%. Similarly, in Brazilian children and adolescents with type-1 diabetes mellitus or with Down's syndrome, the prevalence of undiagnosed and subclinical forms of CD was 4.8% and 5.6%, while the rate of silent cases of gluten intolerance among first degree relatives of celiac patients was 13.66%. Overall, these data on the general population and on groups at risk clearly indicate that the epidemiology of gluten intolerance in Latin America is comparable to that of European and North American Caucasian populations[14].

1.3. Pathology

CD affects the mucosa of the prossimal small intestine with damage gradually decreasing in severity towards the distal small intestine, although in severe cases, the lesion extend to the ileum. The degree of proximal damage varies greatly depending on the severity of the disease. The proximal damage may be very mild in “silent” form with little or no abnormality detectable histologically in the mid jejum. Abnormalities of the gastric and rectal mucosa may be observed in some cases.

The introduction of per-oral endoscopy to take biopsy specimens of the proximal small intestinal mucosa allowed to distinguish between normal villous mucosa and flat biopsy specimens that are present in CD with degrees of abnormalities falling between these two categories.

Normal small intestinal mucosa exhibits digitate villi, leaf forms, and ridges (Appendix I, *Figure 1A*). The villi vary in size, shape, and height but are usually 3 times taller than they are wide. Convolutions, which are normal, are long ridges that can be regarded as villi that have fused and buckled. There are different appearances of the small intestinal mucosa, depending on whether subjects reside in temperature or tropical climate. Fully convoluted appearances occur in more than 5% of normal subjects in tropical areas. Infants exhibit broad leaves and villi, with finger-shaped villi rarely present.

The small intestinal mucosa in CD may be flat and featureless and may present a flat mucosal pattern caused by interaction of deep depressions leaving elevated mounds. Each mound has 8-10 crypt openings.

The characteristic histologic appearance of small intestine mucosa from a patient with untreated CD classically ehibits a flat mucosa with reduction in the normal villous height to crypt depth ration from between 5:1 and 3:1 (Appendix I, *Figure B-D*). The appearance of villous atrophy signals the most severe lesion in CD. The nomenclature describing the various stage or grades is confusing. It includes partial villous atrophy (shortening of the villi), subtotal villous atrophy (i.e. sever villous atrophy or a flat mucosa), and total villous atrophy (i.e. abstent villi) (Appendix I, Figures B-D). In this context Oberhuber *et al.*[72] proposed the following terms: mild atrophy (villous atrophy indicates minor or moderate degrees of shortening and blunting of the villi), marked atrophy (villous atrophy indicates that only short tent-like remainders of the villi are to be seen, flat mucosa or total villous atrophy (it indicates that no more villi are to be recognised and that the surface is flat). The term subtotal villous atrophy was used in the original classification in cases whit a flat mucosa[72]. Today, the term subtotal villous atrophy is often used to describe a marked villous atrophy.

The characteristic patchy lesions may occour especially in relapses and DH. Patchy lesion denotes that parts of the mucosa may appear mormal and others severely diseased. The latter may be missed when only single biopsies are taken.

The total thickness of the mucosa may be increased because of crypt hyperplasia and infiltration of the lamina propria by plasma cells and lymphocytes. Crypt hyperplasia represent the first architectural change which could be found in the dynamic process of the development of the CD. Initially, the elongated crypts are covered by normal appearing villi; later on, when the lesion is more advanced, by shortened villi, which may be absent in the most severe stage. Crypt hyperplasia can only be appreciated on sections cut perpendicular to the plane. Crypt mitotic activity is normally confined to the lower third of the crypt, but in CD this activity my be increased, although the histologic appearance of the crypt seems normal.

Surface enterocytes height is reduced. Especially, in a flat mucosa, the surface epithelium is often cuboidal and basophilic, and the cytoplasm may contain vacuoles.

The histologic differential diagnosis will be between CD, tropical sprue, eosinophilic enteritis and Chron's disease. Confirmation of the diagnosis will include improvement in the histologic abnormalities on the small intestinal biopsy specimen as well as symptomatic improvement with GFD.

There is a generalized lymphocytic infiltration of the epithelium that may extend into the crypts.

The time taken for the cells to migrate from the crypt to the surface is reduced from between 3 and 5 to between 1 and 2 days. The number of IELs in relation to the number of surface cell enterocytes is increased in patients with active disease. Chronic inflammatory cells infiltrate the small intestinal mucosa in untreated CD. There are increased numbers of plasma cells in the lamina propria and lymphocytes in the surface epithelium. Most IELs express the common leukocyte antigen CD3, 70% express the suppressor/cytotoxic phenotype, and 20% of the cells are CD3+, CD4-, and CD8-. There is also an increase in the number of IELs expressing the more primitive λ/δ T-cell receptor in the untreated condition.

According to Marsh[73] the mucosal lesion has been classed into 5 types.

- ***Type 0 preinfiltration lesion***. The duodenal histology is histological indistinguishable from the normal mucosa, but pathological intestinal specimens are able to secrete AGA (IgA and IgM). However it is not true if the duodenal mucosa of these patients remains static or progresses to a more severe injury. This lesion has been described in 5% of patients with gluten-associated dermatitis herpetiformis (DH) (distinguished from linear IgA disease thanks to granula deposits of IgA in dermal papillae).
- ***Type 1 infiltrative lesion***. There is a normal mucosal architecture but with an increased number of IELs This lesion occurs in 40% of patients with DH and in 10% of first degree relatives of patients with CD. This lesion is not associated with any symptoms or signs of malabsorption and intestinal permeability are normal.

- ***Type 2 hyperplastic lesion***. In addition to the increased IELs, there is an increase in crypt depth without a reduction in villous height. Gluten-challenge can induce these changes, which are seen in 20% of untreated DH patients and celiac patients.
- ***Type 3 destructive lesion***. This is the classic CD lesion. It is found in 10% of DH patients, and, 10%-20% of first degree relatives of celiac patients, and, despite marked mucosal changes, many individuals are asymptomatic and therefore classified as subclinical. This lesion is characteristic of, but not diagnostic for, CD and can also be seen with severe giardiasis, infantile food sensitives, graft versus host disease (GVHD), chronic ischemia of the small intestine, tropical sprue, Ig deficiencies, and other immune deficiencies and allograft rejection. This lesion includes entirely the criteria established by intestinal immuno-mediated cell immunity (Table II).
- ***Type 4 hypoplastic lesion***. This can be considered the end-stage lesion in a very small group of patients who are unresponsive to GFD and may develop malignant complications. There is deposition of collagen in the mucosa and submucosa (collagenous sprue), which is usually unresponsive to treatment with steroids, immunosuppressive agents, or chemotherapy.

Table II. *Features of the cell-mediated immunological of the small intestina mucosa*[73].

- Disappearance of intestinal villi
- Crypt hypertrophy
  - Increased rate of cell mitosis
  - Increased rate of cell migration
- Lymphoid infiltration of the epithelium
  - Surface
  - Crypts
- Oedema of the *lamina propria*
  - Increased microvascular hyperpermeability
  - Increased cell population
    - Plasma cells
    - Neutrophils
    - Eosinophils
    - Basophils
    - Mast cells
  - Secretion of cytokines and inflammatory mediators
- Altered profile of differentiation of the membrane hydrolase of the superficial enterocytes
- Upregulation of the expression of class 2 MCH
- Increased fluid secretion
- Increased permeability

Oberhuber *et al.*[72] slightly have modified the classification of Marsh[73].

In Marsh's classification, cases with mild, marked as well as total villous atrophy were embraced by one specification (destructive lesions, type 3), which does not allow one to read these differences when only a code is used. Oberhuber *et al.*, in 1999, have subdivided type-3 lesions into subgroups a-c (Table III).

Tabella III. *Marsh classification modified by Oberhuber et al*[72].

	Type 0	Type 1	Type 2	Type 3a	Type 3b	Type 3c
IEL*	< 40	> 40	> 40	> 40	> 40	> 40
Crypts	Normal	Normal	Hypertrophic	Hypertrophic	Hypertrophic	Hypertrophic
Villi	Normal	Normal	Normal	Mild atrophy	Marked atrophy	Absent

* Numbers as given as IELs/100 epithelial cell

The severity of CD is distinguished as follow:

Type 0. This is a normal mucosa with less than 40 IELs/100 enterocytes.

Type 1. This is the infiltrative type, which is characterised by a normal villous architecture, a normal height of the crypts and an increase in IELs, number up to more than 40 IEL/100 enterocytes. This type of lesion may be observed in CD patients on a GFD and indicates that minimal amounts of gliadin are still ingested or that the patient is not yet in full remission. More importantly, it is also found in some family members of CD patients (potential CD, see later). This stage is not diagnostic for CD and, currently, these patients do not have to undergo a GFD, even if they produce EMA. However, they will have to be followed up for years, because the changes from type-1 to flat mucosa may occur at any time within several years. IELs counts may fall with gluten exclusion and rise on its re-introduction.

Type 2. This is the hyperplastic type, which is characterised by a normal villous architcture, an increase in IELs up to more than 40 IELs/100 enterocytes and crypt hyperplasia. This stage is only rarely encountered; it could be observed in patient on a GFD having a patchy lesion or in individuals who have not yet developed full blown disease.

Type 3. This stage signals the so called “destructive” type of the CD lesion. It is divided into three different subgroups depending on the digree of villous atrophy. Type 3 lesions represent diagnostic lesions.

> ***3a.*** Characterised by a mild villous atrophy, crypt hyperplasia and an increase in IELs up to more than 40 IELs/100 enterocytes.
>
> ***3b.*** Characterised by a marked villous atrophy, crypt hyperplasia and an increase in IELs up to more than 40 IELs/100 enterocytes.
>
> ***3c.*** Characterised by a flat mucosa, crypt hyperplasia and an increase in IELs up to more than 40 IELs/100 enterocytes.

Type 4. This is the very rare hypoplastic lesion, which is characterised by a flat mucosa with normal crypt height and normal IELs counts.

1.4. Etiology

1.4.a. Gluten and peptides

CD could may be defined as a condition in which there is an abnormal proximal small intestinal mucosa that improves morphologically on treatment with a GFD and relapses when glutan is reintroduced.

Gluten is the protein fraction of most cereals, including wheat, rye, and barley.

In this context, gluten collectively refers to prolamine of wheat, rye, barley and oats, the latter still being a matter of controversy, since recent studies seem to exclude its pathogenetic role[69,74-77].

The precise structure of the part of gluten, which cause damage in CD, remains unclear. Wheat grains have 3 major constituents that are separated by milling: the outer husk or bran, the germ, and the endosperm or white flour, which constitutes 70%-72% of the whole grain by weight and contains the toxic components (*Figure 3*).

The storage proteins of cereals fall into 2 major groups: the ethanol-soluble fraction termed prolamins and the glutenins (alcohol-insoluble)[69]. Prolamins from the different cereals are termed gliadins from wheat, secalins from rye, hordeins from barley, avenins from oats, and zeins celiac non-toxic maize.

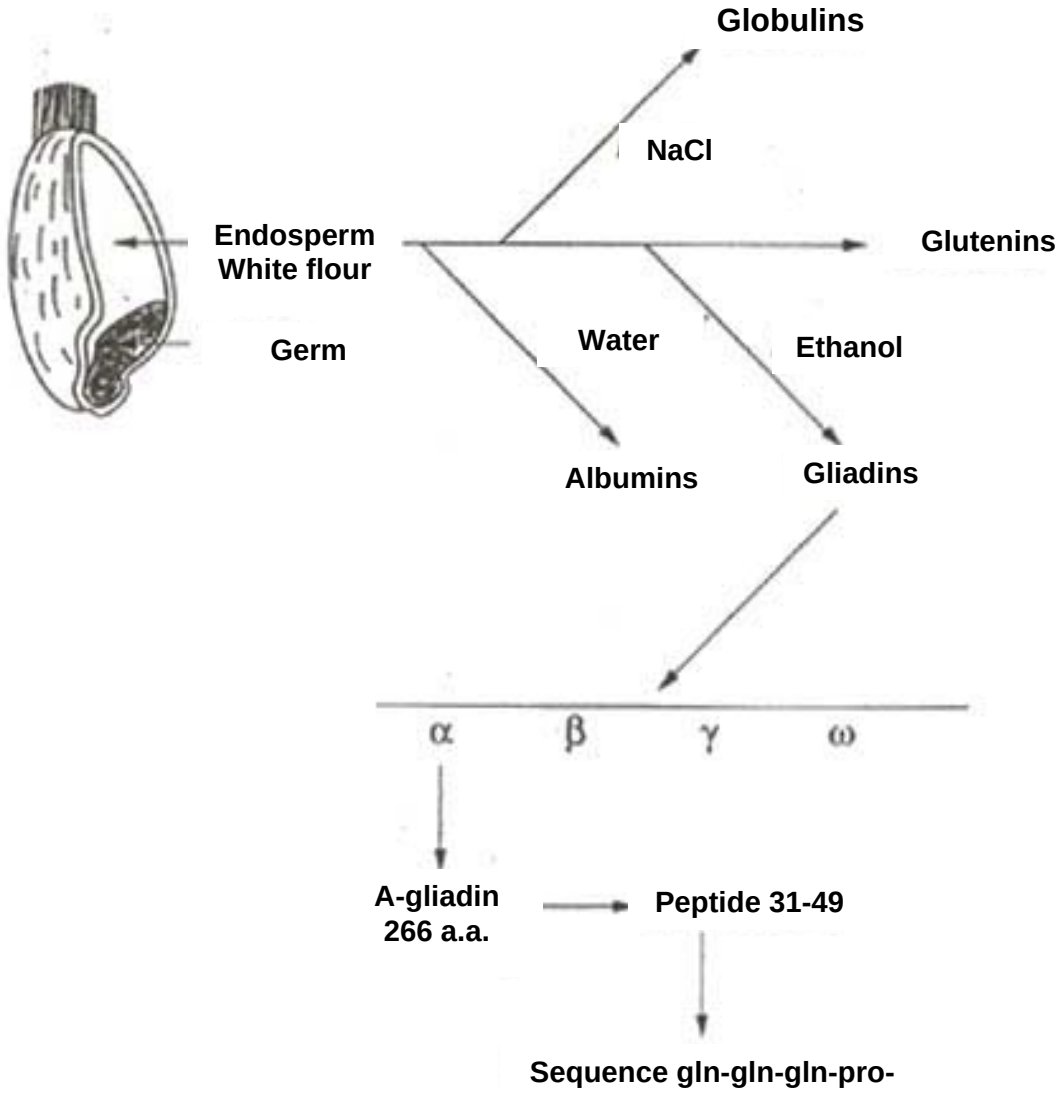
Wheat proteins are divided into classes according to their solubility characteristics: gliadins are soluble in 40-90% ethanol, and glutenins are insoluble in neutral aqueous solution, saline, or ethanol. The gliadins may be further subdivided into alpha, beta, gamma and omega subfractions either according to their relative electrophoretic mobility, or alpha, beta, and omega according to their N-terminal amino acid sequences.

Although there is recent evidence that even glutenins could be involved in the pathogenic mechanisms of CD[78,79], prolamins (namely, gliadins, secalins, and hordeins, respectively) are thought to be responsible for triggering CD.

One of the known toxic α-gliadins, A-gliadin, has been sequenced and comprises 266 aminoacids divided in five domains[69]. A number of *in vitro* studies have suggested that sequences derived both from the N-terminal and C-terminal region of this fraction may be coeliac activating. For A-gliadin 31-49, these data were also confirmed by *in vivo* challenge experiments[80]. Alanine substitution at position P38 of sequence 31-49 resulted in an increased DQ2-binding affinity but in a loss of toxicity[81] which could be of great interest in terms of oral tolerance induction. Moreover, a peptide corresponding to residues 206-217 of A-gliadin and with a high grade of homology to the EIB protein of Adenovirus 12, an adenovirus usually isolated from the GT, was found to be toxic when directly instilled into the coeliac intestinal mucosa[82]. Although the adenovirus hypothesis was attractive and has analogies to theories of the pathogenesis of autoimmune disease, data about this topic have not been universally confirmed[83]. Finally, partially overlapping sequences in the region 57-75 of α-gliadin seem to have all the characteristics to be considered possible candidate

epitopes. In fact, they are recognised by intestinal DQ2-restricted T-cells and act as strong antigens after tTG deamidation at position Q65[84-86]. This deamidation leads to the exposure of negatively charged aminoacids that are required for binding to the groove of HLA class II molecules. Preliminary studies have also demonstrated the toxicity of this sequence to the jejunal coeliac mucosa using an organ culture assay[69].

Figure 3. – *Schematic presentation of wheat corn.*



A high content of glutamine and proline is a common feature of gliadins, secalins and hordeins, while prolamins of cereals considered to be non-toxic for persons with CD, such as rice and corn, have a lower content of these aminoacids[87]. This particular aminoacid composition confers resistance to complete degradation by gastrointestinal proteolytic enzymes, which results in accumulation of peptide fragments rich in glutamine and proline in the lumen of the small intestine[88]. An exceptionally immunoreactive 33-mer peptide LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (residues 57–89) resistant to digestion by all gastric, pancreatic, and intestinal brush-border membrane proteases has been

identified from α-2 gliadin[89], and an *in silico* analysis of the gluten proteome has led to the identification of as many as 60 putative peptides that have similar characteristics[90]. The 33-mer peptide is of notable interest not only for its exceptional resistance to proteolytic degradation, but importantly, to the presence in it of three distinct T-cell epitopes that were identified previously in T-cell proliferation assays, namely, PFPQPQLPY, PQPQLPYPQ (three copies), and PYPQPQLPY (two copies), all recognized by DQ2[91]. This peptide is considered a celiac "superantigen.

Finally, more recently described is a 25-mer containing another well studied fragment of the a-gliadin molecule, p31–43[92].

In conclusion, at least five different epitopes have been identified to date[93]. Three of them are DQ2-restricted and deamidation of a single glutamine residue is essential for lymphocyte activation[94]. Two are DQ8-restricted epitopes[95] one gliadin- and one glutenin-derived, and tTG-mediated deamidation is relevant in one case only[96]. The existence of several gluten epitopes that are recognised by intestinal T cell clones is not contradictory, but it might be explained by the epitope spreading[97]. However, it is still unknown whether the peptides identified, so far, are also involved in the initiation of the gluten specific T cell response. In fact, identification of disease eliciting peptides is going to be important in terms of new strategies of prevention and treatment.

1.4.b. Epithelial barrier dysfunction

For the toxic components of gluten to initiate the cascade of pathogenetic events leading to the histopathologic changes seen in CD (see paragraph 1.3), their translocation into the subepithelial compartment must take place either via the transcellular or by the paracellular routes (or both). Recently, evidence has been provided identifying potential pathways for the transcellular passage of gliadin fractions[91]. This evidence was suggested by *in vitro* transepithelial resistance measurements of epithelial cell monolayers without notable disruption or injury of the cells[98] and by fluorescence-tagged a2-gliadin 33-mer not only in Caco-2 cell monolayers but also in human duodenal biopsy samples of active celiac patients[99]. In this latter system, epithelial translocation of the toxic 33-mer peptides occurs by transcytosis after partial degradation and is regulated by interferon (IFN)-γ. The uptake of this peptide is higher in untreated CD than in controls and in CD patients on a GFD.

In course of CD, if on the one hand it reduces the absorption of nutrients through the intestinal mucosa, secondly, there was an increase in intercellular permeability.

This is due to the opening of tight junctions (TJ), dynamic structures that normally regulate the flow of ions and soluble molecules through intercellular spaces, under the control of a variety of environmental stimuli, physiological and pathological conditions.

In physiological conditions, intestinal epithelium with its intact TJ, acts as a barrier to the passage of foreign antigens, including gluten and bacterial toxins. When TJ are functionally intact, small but significant amount of antigen immunologically cross this barrier through two routes: 90% of the protein absorbed by the gut passes away "trans-cellular" and undergoes lysosomal degradation, which converts them into not immunogenic small peptides. The remainder is transported in the form of intact proteins by "para-cellular" route, i.e. through the TJ, involving a sophisticated regulatory mechanism that leads to antigen tolerance.

When this balance is compromised, as observed during CD, a complex immune response with both an autoimmune and allergic component, can be triggered by various environmental stimuli.

The para-cellular route for the passage of gliadin peptides has also been investigated in particular relation to a peptide, zonulin[100]. It is an intestinal peptide involved in the regulation of TJ, and it seems to be responsible of the increased intestinal permeability observed in CD and of the increased autoimmune disorders observed in untreated CD[101].

This protein is, in fact, known to induce disassembly of the tight junctions and, therefore, is hought to be involved in the early pathophysiological changes that lead to abnormal entry of gliadin peptides in celiac patients.

A new study[102] now finds that gliadin (via at least two a-gliadin 20-mer peptides) binds to the chemokine receptor CXCR3 and this binding results in a stimulation of the release of zonulin, previously demonstrated to be upregulated in celiac mucosa[103]. It is of interest to note that the stimulatory effect of gliadin on zonulin release and on intestinal permeability

can be observed in tissues not only from celiac patients but also, although to a lesser degree, from controls[102,103].

Zonulin by binding to a membrane receptor and in an "organ-specific" way triggers a chain reaction that leads to the rearrangement of the cytoskeleton leading to of signal of opening the TJ and so to the possible entry of allergens and great amounts of gliadin in the intestinal submucosa where, in the presence of genetically susceptible immune cells, it is activates an autoimmune response.

1.4.c. Genetic factors

Genetic predisposition plays a key role in CD and considerable progress has been made recently in identifying genes that are responsible for CD predisposition. It is well known that CD is strongly associated with specific HLA class II genes known as HLA-DQ2 and HLA-DQ8 located on chromosome 6p21. Most CD patients (around 95%) express HLA-DQ2 and the remaining patients are usually HLA-DQ8 positive. The HLA-DQ2 allele is common and is carried by approximately 30% of Caucasian individuals. Thus, HLA-DQ2 or HLA-DQ8 is necessary for disease development, but not sufficient, as its estimated risk effect is only 36–53%[104].

Non-HLA genes contribute more than HLA to the CD genetic background; however, this predisposition depends on a multitude of genes, each of them adding only a modest contribution to disease development. Due to small effect size and genetic heterogeneity between populations, the search for non-HLA CD predisposing genes is like looking for a needle in a haystack. However, this process has been facilitated by the recent application of genome-wide association studies, a hypothesis-free approach that can test thousands of single nucleotide polymorphisms (SNPs) across the whole genome for association[105]. A provisional list of CD predisposing loci includes CELIAC1 on chromosome 6 (HLA-DQ2 and HLA-DQ8), CELIAC2 on chromosome 5q31-33, CELIAC3 on chromosome 2q33 (containing the T-lymphocyte regulatory genes CD28, CTLA4, and ICOS), and CELIAC4 (the myosin IXB gene, MYO9XB) on chromosome 19p13.1.

MYO9B encodes an unconventional myosin molecule that may have a role in actin remodeling of epithelial enterocytes. It has been hypothesized that this genetic variant might lead to an impaired intestinal barrier, which might allow the passage of immunogenic gluten peptides[106]. Although the MYO9B association has not been replicated in some European populations, it was a puzzling finding that MYO9B genetic variants predispose also to inflammatory bowel disease. These data imply shared causal mechanisms underlying intestinal inflammatory diseases[107]. Genetic variation in MYO9B was found to be associated also with systemic lupus erythematosus and rheumatoid arthritis, suggesting that MYO9B is a general risk factor for autoimmunity[108].

Furthermore, associations with tight junction genes PARD3 and MAGI2 has been reported in Dutch patients affected with either CD or ulcerative colitis, again suggesting a common defect of the intestinal barrier in these two conditions[109]. The first genome-wide association studies in a large cohort of UK CD patients and controls identified risk variants in the 1q31, 3q25, 3q28, 12q24 and 4q27 regions. In addition, new evidence for association in the region 2q31 (ITGA4) was reported[110].

The 4q27 region contains IL2 and IL21 genes[111]. IL-2, secreted in an autocrine fashion by antigen-stimulated T cells, is a key cytokine for T-cell activation and proliferation. Another T-cell-derived cytokine, IL-21, enhances B-cell, T-cell, and natural killer cell proliferation and

IFN-γ production. Both cytokines are implicated in the mechanism of other autoimmune conditions, namely type 1 diabetes and rheumatoid arthritis, suggesting that the 4q27 region might represent a general autoimmune disease risk locus.

Furthermore, recent evidences in non-Europeans populations have suggested that rs6822844 at the IL2-IL21 region is strongly associated with multiple autoimmune diseases, such as CD, type 1 diabetes, rheumatoid arthritis, primary Sjögren's syndrome, systemic lupus erythematosus and Behçet's disease in individuals of European descent, suggesting similar molecular mechanisms[112].

A further genome-wide association studies on follow-up samples from three independent European CD collections identified seven previously unknown regions contributing significantly toward disease risk[113]. These seven newly identified regions, together with IL2–IL21, explained 3–4% of the heritability of CD. Six out of seven regions harbored genes controlling immune responses, for example leukocyte signaling in response to IL-18 and IFN-g production. Together with other recent genome-wide association studies reports[114] these findings suggested possible common mechanisms between CD and type 1 diabetes at the SH2B3 region, 3p21 CCR gene region, RGS1 (1q31), IL18RAP (2q12), TAGAP (6q25), PTPN2 (18p11), and CTLA4 (2q33)[115,116] and between CD and Crohn's disease at the IL18RAP region.

Table IV. *Non-HLA loci of CD susceptibility*[117].

Loci identified	Type of study used for identification	Origin of the cohort(s)	Candidate genes (function)
CELIAC 2 5q31-q33	linkage analysis	Italy, Finland, Scandinavia, Europe (meta-analysis)	Unknown
CELIAC 3 2q33	Candidate gene approach	France, The Netherlands, Sweden, Norway	CTLA4 (T cell response)
CELIAC 4 19p13.1	linkage analysis	Netherland	Myosin IXB (Rho family guanosine triphosphatase)
CELIAC 5 15q11-q13	linkage analysis (microsatellite)	Finland	Unknown
CELIAC 6 4q27	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Italy, United States, Scandinavia	KIAA1109 TENR (ADAD1) IL2 IL21
CELIAC 7 1q31	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Italy, United States	RGS1 (B-cell activation)
CELIAC 8 2q11-q12	GWAS (SNPs)	United Kingdom, Netherland, Ireland	IL18RAP IL18R1
CELIAC 9 3p21	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Spain	CCR1 (chemokines) CCR2 CCRL2 CCR3 CCR5 XCR1
CELIAC 10 3q25-q26	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Italy, United States	IL12A
CELIAC 11 3q28	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Italy, United States	LPP (zinc binding protein)
CELIAC 12 6q25.3	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Italy	TAGAP (T cell activation)
CELIAC 13 12q24	GWAS (SNPs)	United Kingdom, Netherland, Ireland, Italy, United States	SH2B3 (TLR intracellular adaptor, T-cell activation)

GWAS, genome-wide association study; SNP, single nucleotide polymorphism.

Two novel CD risk regions were identified at chromosomes 6q23.3 (OLIG3-TNFAIP3) and 2p16.1 (REL) by testing large case-control European cohorts, both genes are key mediators in the nuclear factor kappa B pathway, an innate immunity that has not been reported to be heritably altered in CD before[118]. To summarize, it appears that the genetic predisposition to celiac disease depends on one gene with a large effect (HLA-DQ2/DQ8) on the adaptive immune response to gluten peptides and many other genes influencing different aspects of innate and adaptive immune reactions, intestinal permeability, and general predisposition to autoimmunity[118].

1.4.d. Environmental factors

Environmental factors that have an important role in the development of CD have been suggested by epidemiologic studies[119]. These include a protective effect of breast-feeding[120] and the introduction of gluten in relation to weaning. The initial administration of gluten before 4 months of age is associated with an increased risk of disease development, and the introduction of gluten after 7 months is associated with a marginal risk (Norris *et al.*, 2005). However, the overlap of gluten introduction with breast-feeding may be a more important protective factor in minimizing the risk of CD.

Recently, a high frequency of rotavirus infections has been shown to increase the risk of CD in genetically predisposed children[121]; in addition, a subset of serum anti-tTG IgA antibodies of persons with active CD has been found to recognize the rotavirus protein VP-7, suggesting a rotavirus driven mechanism of molecular mimicry in the pathogenesis of the disease[122].

1.5. Pathogenesis

As seen in paragraph 1.4.a. prolamins of gluten are rich in proline and glutamine, and undergo partial but incomplete digestion in the small intestine. The result is an accumulation of relatively large peptide fragments, as many as 50 aminoacids in length (such as a recently described 33mer sequence in CD)[89,90].

Under normal physiologic conditions, the intestinal epithelium, with its intact intercellular TJ, serves as the main barrier to the passage of macromolecules, such as gluten proteins. Through the possible mechanisms seen in paragraph 1.4.b. large peptides are able to cross the epithelial barrier and reach antigen-presenting cells in the *lamina propria* stimulating an immune response.

Data from Maiuri and colleagues clearly show that certain gluten peptides elicit an innate immune response, while others drive adaptive immunity[123-126].

The adaptive immune response involves CD4+ T cells in the *lamina propria* that recognize specific immunogenic gluten peptides processed and presented by antigen-presenting cells[127]. Gluten antigens are modified enzymatically by tTG2, a calcium dependent which is expressed by many cell types and associates with the extracellular matrix (endomysium or reticulin fibers). TG2 targets certain glutamine residues in some extracellular and intracellular proteins, usually tethering them to a lysine residue of a second protein that results in crosslinking of both proteins. Alternatively, in an acidic pH that occurs with inflammation, TG2 merely deamidates these glutamines to negatively charged glutamic acid residues[128]. Due to their high content in glutamine and neighboring proline and hydrophobic aminoacid residues, gluten proteins, especially prolamins but also the glutenins, are preferred substrates for TG2[117].

These deamidated peptides are more antigenic than native gluten peptides and usually adhere to the binding grooves of HLA-DQ2 and HLA-DQ8 molecules with a higher affinity than native peptides[129]. HLA-DQ peptide complexes trigger a more rigorous gluten-specific CD4+ Th1 T cell activation, which in turn stimulate production of autoantibodies in the form of anti-tTG and EMA[130]; the presence of these antibodies is a specific indication of CD.

Subsequently, CD4+ T lymphocytes infiltrate the *lamina propria*, activated CD4+ T-cells release cytokines (such as IFN-γ) that promote immuno-inflammatory mechanisms (such as secretion and activation of matrix metalloproteinases by fibroblasts and and inflammatory cells) that ultimately lead to tissue damage[131]. Activated CD4+ T-cells also induce activation and clonal expansion of B-cells, which produce antibodies: AGA, EMA, and anti-tTG. Production of antibodies directed against tTG is probably due to the fact that tTG can form covalent complexes with gluten peptides[132]. The role of these antibodies in the pathomechanism of CD is unclear; nonetheless, they are important for diagnostics[127,133]. *Figure 4* summarizes pathogenesis of CD.

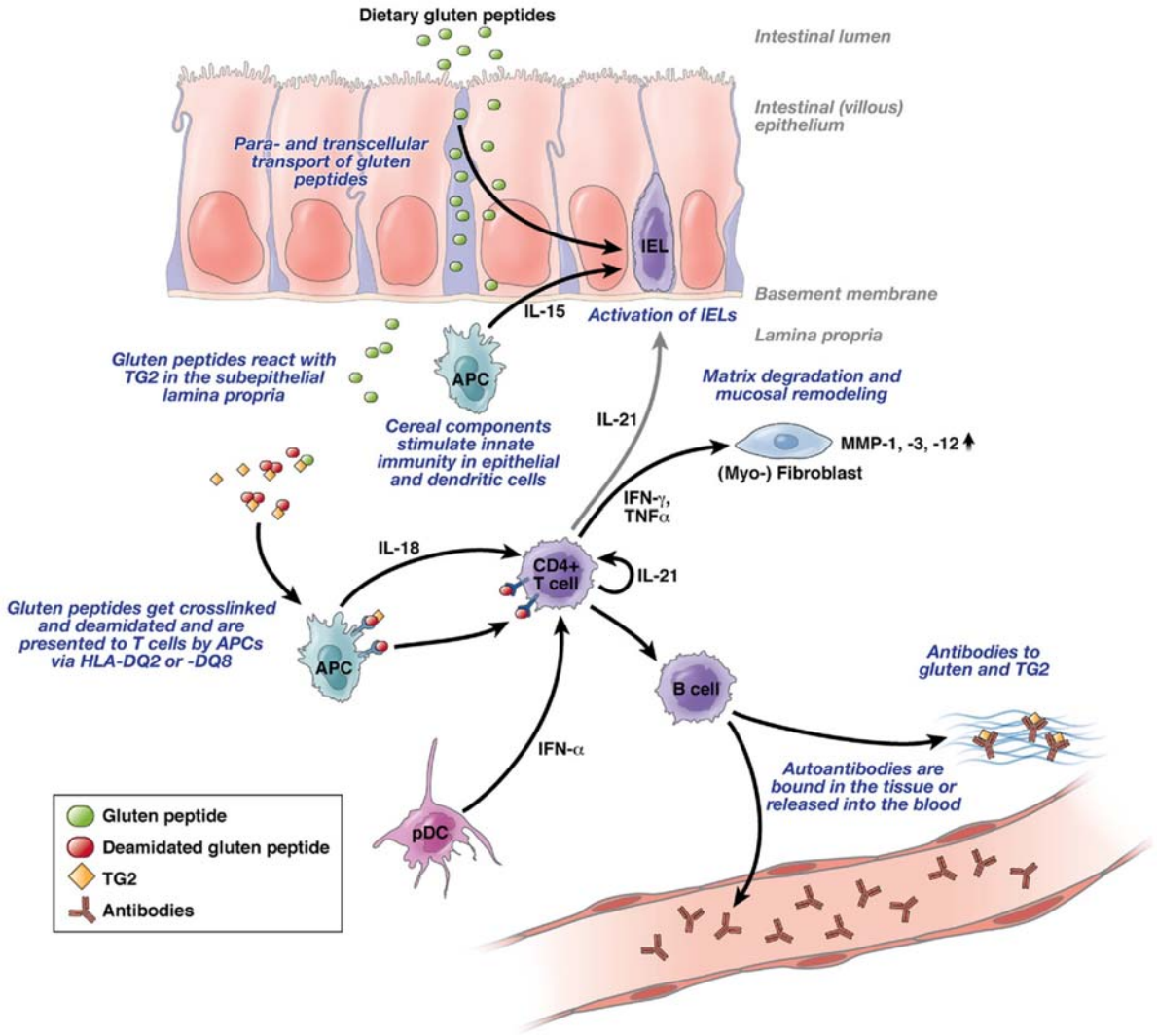


Figure 4. *Pathogenesis of CD. Gluten peptides that are highly resistant to intestinal proteases reach the lamina propria, via either epithelial transcytosis or an increased epithelial TJ permeability. Cross-linking and particularly deamidation of gluten peptides by TG2 creates potent immunostimulatory epitopes that are presented via HLA-DQ2 or HLA-DQ8 on antigen-presenting cells. Subsequently, CD4+ T cells are activated, secreting mainly Th1 cytokines such as IFN-γ, which induces the release and activation of metalloproteinases (MMPs) by myofibroblasts, finally resulting in mucosal remodeling and villus atrophy. Additionally, Th2 cytokines are produced driving the production of (auto-)antibodies to gluten and TG2. Other cytokines such as IL-18, IFN-γ, or IL-21 seem to play a role in polarizing and maintaining the Th1 response. Furthermore, IL-15 links the adaptive immune system to innate immune responses (see Figure 5). The scheme is simplified. It does not show that T cells circulate to mesenteric lymph nodes where they encounter and are primed by antigen-presenting cells (mainly dendritic cells) and from where they home back to the lamina propria, a process that is driven by the lymphocyte homing receptors CCR9 and integrin α4β7*[117].

Although the importance of the adaptive immune response to gluten has been well-established, observations now also point toward a central role for the gluten-induced innate stress response in the pathogenesis of CD and its malignant complications[134].

Proteins from wheat, rye, or barley (apparently in contrast to “non toxic” cereal proteins derived from, for example, corn or rice) can elicit an innate immune response in professional antigen-presenting cells (monocytes, macrophages, and dendritic cells) that activates

predominantly IELs, but also intestinal epithelial cells[123,135-138]. *Figure 5* summarizes innate immune response.

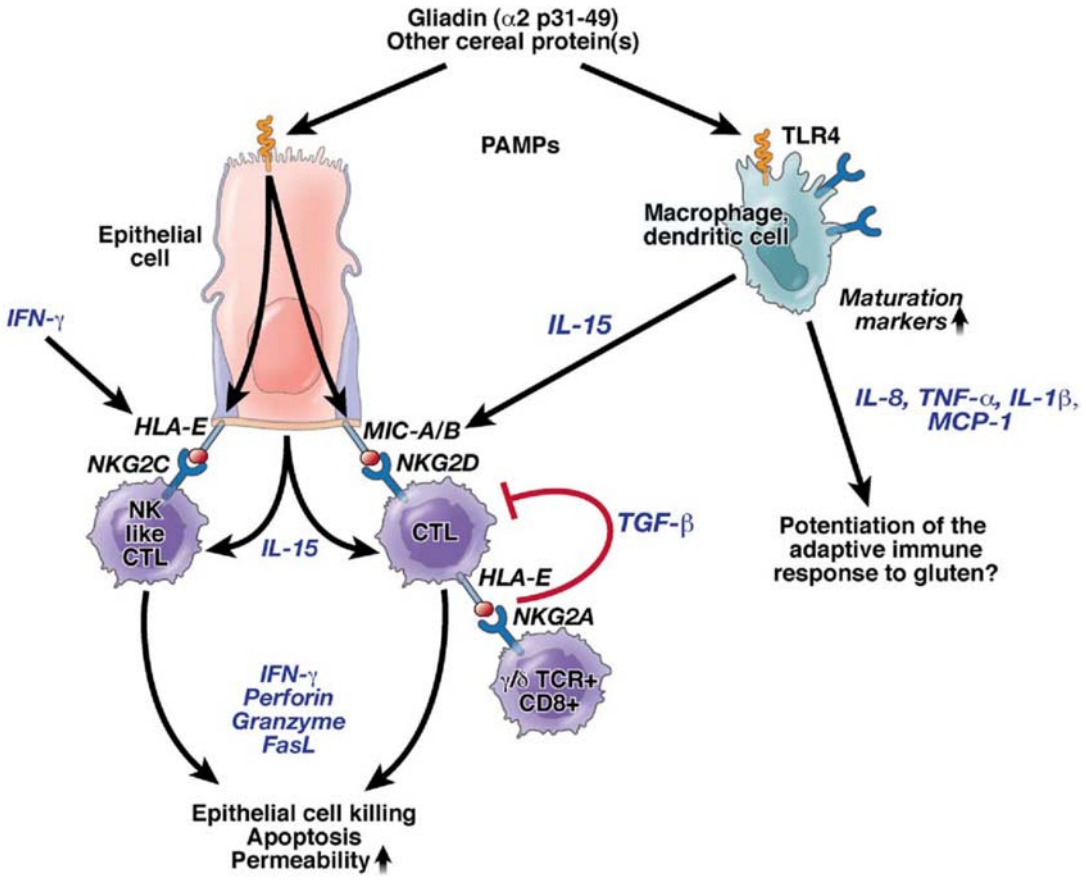


Figure 5. *Innate immune responses CD. Upon stimulation with gliadin peptide p31-49 (and other peptides), epithelial cells, macrophages, and dendritic cells secrete IL-15, which in turn up-regulates both the NKG2D receptor on IELs and its epithelial ligand MICA. The thus stimulated cytotoxic lymphocytes induce increased epithelial apoptosis and permeability. Furthermore, the NKG2C receptor on a subset of natural killer–like IELs is stimulated by its epithelial ligand HLA-E on epithelial cells, resulting in their proliferation and cytotoxicity, whereas stimulation of γ/δ CD8+ IELs bearing the NKG2A receptor via HLA-E induces TGF-β secretion and therefore a regulatory phenotype. Gliadin (cereal) peptides can also directly elicit innate immune responses in macrophages and dendritic cells via pattern recognition receptors such as Toll-like receptor 4 or other MyD88-dependent pathways. This drives maturation of these cells and secretion of inflammatory cytokines such as IL-1β, IL-8, tumor necrosis factor α and MCP-1, which can potentiate the adaptive immune response to gluten.*
APC, antigen presenting cell; pDC, plasmacytoid dendritic cell.[117].

This innate immune response is an immediate reaction and is usually directed against relatively uniform microbial antigens but also against yet ill-defined constituents of cereals[139]. In CD, the innate immune response appears to favor the development of adaptive immunity to gluten in patients that carry HLA-DQ2 or HLA-DQ8[123]. α2-gliadin peptide p31-43, which is distinct from peptides that elicit adaptive immunity, was shown to trigger innate immunity in intestinal epithelia and intestinal organ cultures[123,140]. Other

peptides reportedly stimulated rodent monocyte or macrophage cytokine release[135,136,138]. However, these peptides have not been generally confirmed and none of the studies identified a receptor on a responsive cellular subset. Two recent studies that rigorously ruled out contamination by lipopolysaccharide implicated MyD88, the major downstream signal transducer of Toll-like receptor 4 on monocytes, macrophages, and dendritic cells, and Toll-like receptor 4 itself as primary receptor for innate responses to cereal proteins[137,141].

The innate immune response is the initial activation step induced by α-gliadin peptides and is mediated primarily by CD8+ T cells, enterocytes, macrophages, and dendritic cells. These cells trigger production and proliferation of proinflammatory cytokines, interleukin-15, which induce expression of natural killer receptor NKG2D and its ligand MIC molecules on epithelial cells that arm the cytolytic NKG2D pathway to destroy stressed epithelial cells. Infiltration by CD4+ T lymphocytes into the lamina propria and CD8+ T lymphocytes into the intestinal epithelium are characteristic of active CD[142].

As regards IELs many progress has been made in our understanding how these cells are activated by luminal cereal proteins. The perforin/granzyme and/or Fas/FasL pathways are central to the observed cytotoxicity and apoptosis-inducing activity of IELs on the intestinal epithelium in CD[143-145]. Innate immune activation of IELs by gluten induces expression of the non-classic class I molecule MICA on the intestinal epithelium, which serves as ligand for the heterodimeric NKG2D receptor on natural killer, γ/δ T cells and on subsets of CD4+ and CD8+ T cells[146]. Epithelial MICA and up-regulated epithelial production of interleukin (IL)-15 leads to activation of NKG2D on IELs[142]. NKG2D also links innate and adaptive immunity, because it both triggers antigen-specific lymphocyte–mediated cytotoxicity and induces a direct cytolytic function independent of T-cell receptor specificity in effector CD8 T cells[147]. Similarly, the NKG2C receptor that is activated by its epithelial ligand HLA-E is implicated in the pathogenesis of CD, stimulating IEL proliferation and cytokine secretion in patients with CD[148-150]. IELs can also have an immunoregulatory capacity through the secretion of transforming growth factor (TGF)-β1, as reported for a subset of CD8+TCRαβ+ IELs that express the inhibitory NK receptor NKG2A. Interestingly, this subset of regulatory cells was reduced in duodenal biopsy specimens from patients with active CD as compared with controls or patients on a GFD[151].

The central role of IL-15 in the activation of innate and adaptive immunity in CD has been confirmed by several investigators[144,152-155], coupled with an increased expression of IL-15 receptor and a lower threshold for activation on IELs[153]. Both intestinal epithelia and dendritic cells/macrophages are major sources of IL-15[156].

Apart from being a potent growth factor for IELs, IL-15 blocks Smad3-dependent transcription via the activation of c-Jun-N-terminal kinase and phosphorylation of c-jun and thus counteracts the immunosuppressive TGF-β pathway. Recently, IL-21, which is

produced by CD4+ Th1 T cells, has emerged as an additional driving force of innate immunity that often acts in concert with IL-15[157].

1.6. Clinical picture

1.6.a. Clinical classification

The clinical presentation of CD varies greatly, depending on a patient's age, the duration and extent of the disease, and presence of extraintestinal manifestations.

The recognition of the celiac iceberg demonstrates the clinical variability of CD and helps to understand its systemic nature (*Figure 6*).

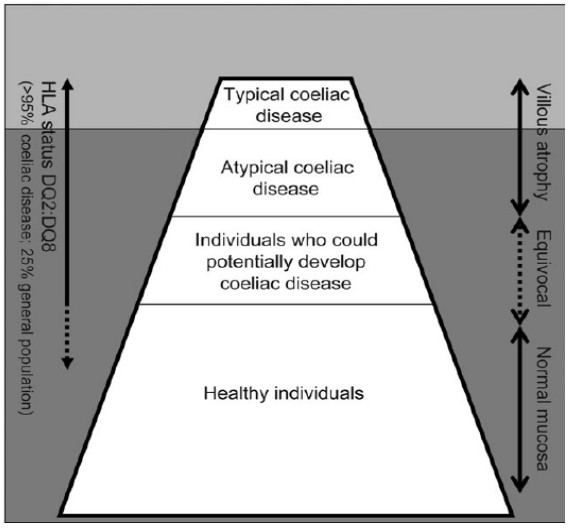


Figure 6. *The "coeliac iceberg"*[158].

The highest stratum of the iceberg (above the waterline) describes patients with typical symptoms that are gastrointestinal. Classic symptoms associated with CD in children are diarrhea, abdominal distension, and failure to thrive[18]. Similarly, adolescents and adults present with diarrhea, constipation, weight loss, weakness, short stature, flatus, abdominal pain, and vomiting[159-161]. Presence of obesity does not exclude the diagnosis of celiac disease[158].

The subsequent stratum (just under the water) contains those patients considered to have atypical presentations of CD (no gastrointestinal symptoms). Atypical clinical manifestations of celiac disease are characterized by few or no gastrointestinal symptoms, instead, extraintestinal symptoms such as iron-deficiency anemia, reduced bone mineral density, chronic fatigue, irritable bowel, dyspepsia, infertility, miscarriage, hypertransaminemia, coagulopathy, short stature, pubertal delay, arthralgia, folate/zinc deficiency, DEDs, SAR or better ALU (see paragraphs 2..1.a-2.2.a) and otherwise unexplained neurological disorders (primarily peripheral neuropathy and ataxia) predominate[162,163].

The third level of this iceberg include latent and silent forms. Latent form refers to patients who were previously diagnosed with CD but who are currently asymptomatic with normal histology despite continued ingestion of gluten. "Latent" may also apply to patients with normal histology on a GFD who will subsequently develop CD due to genetic predisposition

or underlying immunologic abnormalities; in this setting, the term "potential" may also be used. Patients with latent or potential CD may or may not have positive serology, and up to 50% of these individuals may develop overt CD[164].

There is also a subset of patients who have asymptomatic or silent form, in which patients have histologic changes, probably limited to the proximal intestine, and positive serologic findings, but are are apparently asymptomatic without signs and symoptoms of malabsorption.

Patients who have a subclinical form of CD are unaware of it because outwardly they have no physical symptoms, yet they have positive serological test results for CD and villous atrophy on intestinal biopsy. Subclinical CD is identified as a result of screening at-risk groups or by recognition on biopsies obtained for another indication (ie, dyspepsia or reflux). Table V shows the possible clinical manifestations of CD.

Table V. *Possible clinical manifestations of CD.*

Typical symptoms	Atypical symptoms	Associated conditions	Complications
Chronic diarrhea	Sideropenic anaemia Vitamin B12 deficiency Folate deficiency	IgA deficiency	Refractory sprue
Failure to thrive	Short stature	Dermatitis herpetiformis	Enteropathy-associated T-cell lymphoma
Abdominal distension	Osteopenia/osteoporosis	Type 1 diabetes	Carcinoma of the oropharynx, esophagus, and small bowel
Steatorrhoea	Alopecia	Autoimmune thyroid diseases	Ulcerative jejunoileitis
Wheight loss	Psoriasis	Sjogren's syndrome	Collagenous sprue
Irritability	Recurrent abortions	Microscopic colitis	
Anorexia	Hepatic steatosis	Rheumatoid arthritis	
	Recurrent abdominal pain	Down's syndrome	
	Gaseousness	Turner syndrome	
	DEDs	Williams syndrome	
	ALU	Congenital heart defects	
	Atrophic glossitis	Addison diseae	
	Ataxia	Autoimmune hepatitis	
	Primary biliary cirrhosis	Autoimmune atrophic gastritis	
	Hepatic steatosis	Autoimmune emocytopenic diseases	
	Hypertransaminasemia	Sarcoidosis	
	Myastenia gravis	Cystic fibrosis	
	Recurrent pericarditis	Fibrosing alveolitis	
	Polyneuropathy	Lung cavities	
	Epilepsy (with or without intracranial calcifications)	Pulmonay hemosiderosis	
	Vasculitis	Inflammatory bowel disese	
	Dilatative cardiomiopathy	Systemic	

		erythematous lupus
	Hypo/hyperthyroidism	Polymiositis
	Follicular keratoses	
	Pre-term	
	Reproductive disorders	
	Thrombocytosis	
	Arthralgia or arthropathy	
	Anxiety and depression	
	Delayed puberty	
	Dysmenorrhea or amenorrhea	
	Vitamin K deficiency	
	Schizophrenia	
	Dyspepsia	
	Reflux	

1.6.b. Clinical presentation

CD primarily affects the proximal small intestine, but can involve the entire small intestine in some individuals. This proximal location of CD often results in overt malabsorption of vitamins and minerals. Diarrhea in CD presentation is due to progression of the disease into the distal small bowel.

Currently, CD is thought to resemble a multisystem immunological disorder rather than a disease restricted to the GT[160]. In fact, The wide range of the CD clinical manifestations must induce many different specialists - gastroenterologists, endocrinologists, rheumatologists, haematologists, cardiologists, neurologists, as well as paediatricians, dermatologists, and dentists[18] - to consider this disorder when a patient presents some clinical signs and symptoms.

Studies confirm[18,161,165] that fewer patients present with severe gastrointestinal symptoms, and the clinical face of CD in the United States is diverse. Adult presentations are now more common than pediatric presentations[18]; and 25% of newly diagnosed CD occurs in patients older than 60 years of age[159]. Subclinical or atypical presentations are more frequently encountered[165,166], and more cases are being diagnosed as a consequence of widespread serological testing and increased awareness.

Infancy and childhood

The onset of symptoms in the classic form generally occurs between 6 and 18 month of age. This form is typically characterised by chronich diarrhea, failure to thrive, anorexia, abdominal distension, apathy, pallor, and muscle wasting. Growth is usally normal during the first month of life. Symptoms begin within weeks to a few months after introduction of weaning foods containing prolamines, and soon there is a progressive decrease in weight gain with a decline in the child's percentile for weight and weight for height. On examination, the children are often pale and noticeably thin with a protuberant abdomen, decreased subcutaneous fat, and reduction in muscle mass. The stools are characteristically pale, loose, bulky, and highly offensive because of fat malabsorpion. Very young infant may present with vomiting, which is often effortless, of large volume, and usually associated with abdominal distention and little or no diarrhea. Abdominal pain may be so severe that a laparotomy is undertaken because a mistaken diagnosis of intestinal obstruction. In some cases, this pain is caused by constipation. Retardation of motor activity has been observed. In the very young infant with early onset of symptoms there may be frank watery diarrhea with dehydratation and electrolyte imbalance. A small number of these infants also have severe hypoproteinemia and oedema and may present in shock-like state tha has been termed "celiac crisis".

Patients with severe, untreated CD may present with short stature, pubertal delay, iron and folate deficiency with anemia, and rickets.

Atypical CD is usually seen in older children and adolescents, who have no overt features of malabsorption. They tend to have more varied and extra-intestinal symptomatology presenting with anemia, failure to grow normally and neurologically symptoms. In fact, in addition to recurrent abdominal pain, hypertransaminasemia, ALU. DEDs, dental eruption delay, arthralgia, children may have behavioural disturbances such as depression, may be irritable, and may perform poorly in school[167].

Laboratory signs of the malabsorption include iron deficiency anemia, hypoalbuminemia, hypocalcemia and vitamin deficiencies.

Adult life

The diagnosis of CD is increasingly being in adults. About 20% of cases occur in patients who are older than 60 years of age[167]. Some patients are short or have symptoms dating back to childhood. However, many have no history of symptoms, suggesting that CD can develop in adulthood. CD may present during pregnancy, or post partum, and the diagnosis should be considered in pregnant women in whom severe anemia develops. In a group of patients diarrhea could be the main presenting feature; however, approximately 50% of adult CD patients does not have significant clinically diarrhea but unspecific signs and symptoms, such as lassitude, loss of weight, unspecific atrophic glossitis, and symptoms of anemia. In fact, iron-deficiency anemia is now the most common clinical presentation in adults with CD.

A small group present because of abnormal psychological or psychiatric symptoms, including schizophrenia. There may be presentation with problems related to osteomalacia, including spontaneous fractures, and myopathy, skin complaints, bleedin diathesis, or infertility.

Many individuals have long-standing ill health for many years and, never having had severe symptoms, accept this as normal. Abdominal pain occurs in 5% of cases. ALU may be the sole presenting symptom, and therefore CD should be excluded in cases of severe unexplained mouth ulceration.

Presenting symptoms in many patients with CD are non-specific and therefore a high diagnostic suspicion should be aroused with minor abnormalities such as haematological or biochemical profiles, including persistent transaminitis.

Because the proximal small intestine is the predominant site of inflammation and also the site of micronutrient absorption, deficiencies of iron, folate, and calcium are common. As the disease progresses along the intestine, malabsorption of carbohydrates, fat, and fat-soluble vitamins A, D, E, and K, and other micronutrients occurs[17]. The finding of a mild unexplained anemia with persistently low serum or red cell folate, iron and vitamin B12 levels should suggest further investigation and may lead to a diagnosis of CD. In fact, the association of CD to refractory iron deficiency anemia is well-established[168-170]. Frequency of iron-

deficiency anemia in CD varies from 12% to 69%[171], and is reportedly higher in patients with long-standing, untreated disease[172]. Incidence of vitamin B12 deficiency in untreated patients ranges from 8% to 41%[172,173], though there is a relative sparing of villous atrophy in the ileum where vitamin B-12 is absorbed. While the mechanisms inducing deficiency are unclear, mucosal changes are considered the most likely cause of vitamin B12 deficiency. Folic acid is absorbed in the jejunum, the proximal segments of which can be inflamed and damaged in active CD.

Atrophic glossitis, angular stomatitis and cheilosis may be present. Nause and vomiting are common, particularly during episodes of diarrhea in children. There may be anorexia, although appetite may be increased in some patients and polyphagia has been reported. Abdominal distention is common and often associated with flatulence.

Symptom of cramp and tetany may occur, usually associated with low serum calcium or magnesium levels.

Bowel disturbance is the most frequent problem, usually in the form of loos stools. The motion may be paler than normal, sometimes offensive, occacionally frothy, and difficult to flush away. Bowel frequency varies, but is commonly 3-4 times a day and rarely more than 8 times a day. Normally formed and colored stools do not preclude the diagnosis. Steatorrhea may be present even though the patient has stools with normal appearance. Physicians should remember to enquire specifically about a subject's stool because patients with long-standing bowel disturbance frequently make no complaint. In addition, clinicians should examine patients' stools to avoid any problem any problem pertaining to the patients' powers of observation. It has been reported that many adults present with episodic or nocturnal diarrhea, flatulence, and weith loss[174].

Secondary lactose intolerance resulting from decreased lactase production by the damaged villi is also common[175].

Abdominal discomfort and bloating are common and often lead to a mistaken diagnosis of irritable bowel disease. In fact, a study has shown that 10% of patients suffering from irritable bowel syndrome were subsequently found to suffering from CD[176]. Constipation has been reported to occur in 10% of cases, although this may be underestimate.

Abdominal pain is uncommon and may be indicate the need for investigation to exclude intra-abdominal pathology complicating CD. In particular such conditions as volvulus, intussception, mesenteric adenitis, cholelithiasis, peptic ulceration, and pancreatitis. Scurvy and epistaxis have been reported, and occasionally there may be bleeding into skin and subcutaneous tissue. This may reflect vitamin K deficiency.

Osteomalacia, rickets or bone pain lead to the diagnosis of CD in an appreciable number of subjects reflecting low calcium ond vitamin D absorption.

1.6.c. Associated diseases and complications of unrecognized CD

A number of medical conditions are significantly associated with CD (Table V).

Malignancy

Untreated CD is associated with a number of complications. The best recognized is malignancy. The overall relative risk of all types of malignancy in coeliac patients has probably been historically overestimated. The relative risk is likely to be less than 2-fold when compared with the general population. Although small intestinal lymphoma may have a 50-fold increased relative risk (compared with the general population), this is still a rare condition.

Enteropathy-associated T-cell lymphoma occurs in adults, with the incidence peaking in the sixth decade of life, and is usually at an advanced stage at diagnosis. Symptoms may include malaise, anorexia, weight loss, diarrhea, abdominal pain, and unexplained fever. The development of lymphoma is usually indicated by clinical relapse of symptoms of CD after a period of good response to gluten withdrawal[119]. Enteropathy-associated T-cell lymphoma usually develops in the jejunum but may also be found in the ileum or in extraintestinal sites (e.g., liver, brain, chest, and bone) and is often multifocal. The prognosis is poor; less than 20% of patients survive for 30 months[177]. The phenotype of enteropathy-associated T-cell lymphoma is consistent with a tumor that derives from a clonal proliferation of intraepithelial lymphocytes. Immunohistochemical phenotyping indicates that this lesion is most commonly CD3+, CD4–, CD8–, CD30+, and CD103+[178].

Thus, the overall number of cases and absolute risk remains low. Improvements in small intestinal imaging modalities such as wireless capsule endoscopy may help to target highrisk individuals such as those with refractory symptoms or ulcerative jejunitis.

Other associated malignancies include oesophageal (proximal and distal), small intestinal adenocarcinoma and colonic cancer[179-181].

In patients with celiac disease, the risk of adenocarcinoma of the small intestine, generally a rare cancer, is increased many fold as compared with the risk in the general population[182]; still, the overall risk is very low given the rarity of this cancer. These carcinomas are most often located in the jejunum and are more likely to develop as an adenoma–carcinoma sequence than as dysplasia in flat mucosa.

Intriguingly, patients with CD may have lower rates of breast and lung cancer[179]. Risk of cancer appears to reduce over time, particularly in patients who adhere strictly to the GFD. Prior studies demonstrated increased cancer incidence at all sites and found correlation with the lack of adherence to the gluten-free diet[181]. A confounding factor may be age at diagnosis of CD with older patients appearing to have a higher risk of neoplasia[183].

Bone mineral density

CD is known to be associated with reduced bone mineral density (both osteopaenia and osteoporosis)[184]. The prevalence of abnormal bone mineral density is reported to be around 40% and leads to an increased fracture risk. This increased relative risk is small overall as bone mineral density usually improves (or remains stable) once gluten has been excluded from the diet. Additionally, the excess fracture risk is small overall even when considering elderly patients where the risk is highest and has been calculated to be two to three additional hip fractures per 10 years of follow-up[185]. Potential protective factors include a lower rate of smoking and a higher rate of hormone replacement therapy use in coeliac patients[186].

Autoimmunity

CD is associated with a number of other conditions some of which are autoimmune in origin. In most cases, CD is identified subsequent to the original diagnosis often because symptoms may be misconstrued as due to the original diagnosis. A good example of this is persistent tiredness in patients with thyroid disease[187]. The effect of concurrent CD on other autoimmune diseases has not been well studied as can be seen by the paucity of outcome data in patients with both CD and type 1 diabetes[188].

There have been some studies suggesting that patients who are not compliant with the GFD are more likely to develop other autoimmune conditions. This may be related to the finding that patients with CD have a high prevalence of other organ-specific autoantibodies. Additionally, following a GFD, the level of these antibodies has been found to fall[189]. However, this is a controversial perspective with other studies suggesting that the development of further autoimmune conditions is independent of gluten exposure and may be related to specific HLA haplotypes.

Reproductive problems

Infertility, reduced fertility and an increased risk of an adverse outcome during pregnancy (miscarriage, low birth weight and intrauterine growth retardation) have all been attributed to undiagnosed CD. However, these risks may be less than historically described[190,191].

Sepsis

Functional hyposplenism may occur in up to 30% of patients with CD — for this reason, vaccination with pneumovax and haemophilus influenza type b vaccines are advised. A recent population database study has described a hazards ratio of 2.6 for sepsis and 3.9 for pneumococcal sepsis[192].

Neurological manifestations

An unusual but more frequently recognized presentation of CD is with neurological symptoms. Studies in this area have historically described ataxia and peripheral neuropathy as presenting features for atypical CD[193-195]. However, more recently two groups have also demonstrated subclinical neuropathy being present in patients who were diagnosed with CD (and did not present with neurological symptoms)[196]. Work done in Sheffield has also described the phenomenon of neurological gluten sensitivity—these patients may have positive serology but either minimal or no changes on duodenal biopsy[193]. Treatment with a GFD can arrest or improve neurological symptoms[194]. The underlying pathophysiology is not fully determined but changes in the humoral response have been described[194]. In addition, excess tissue transglutaminase type 2 deposits have been found in duodenal samples in such patients (even when structurally normal at conventional histology)[197]. tTG is ubiquitous and therefore tTG antibodies may themselves be pathogenic in some patients[198].

1.7. Diagnosis

The diagnosis of CD is based on 3 key parameters: (1) case identification, (2) screening using serological tests, and (3) small intestinal biopsy. As demonstrated by the coeliac iceberg, the presence of relevant symptoms is not essential for a diagnosis of CD.

No single test exists that can definitively diagnose or exclude CD in every individual[199]. Serological testing, used as an initial non-invasive screen, is the first step in pursuing a diagnosis of CD. Typical indications for serologic testing include unexplained bloating or abdominal distress; chronic diarrhea, with or without malabsorption or the irritable bowel syndrome; abnormalities on laboratory tests that might be caused by malabsorption (e.g., folate deficiency and iron-deficiency anemia); first-degree relatives with CD; and autoimmune diseases and other conditions known to be associated with GSE[119].

The most sensitive antibody tests for the diagnosis of CD are of the IgA class. The available tests include those for AGA, connective-tissue antibodies (antireticulin and EMA), and antibodies directed against tTG, the enzyme responsible for the deamidation of gliadin in the lamina propria[119].

EMA are autoandibodies directed against the endomysium, a connettive tissue protein found between myofibrils in the GT; whilst the demonstration that the antigen for EMA was tTG has allowed the development of an enzyme-linked immunosorbent assay for both IgA and IgG tTG to screen CD[200].

The AGA are no longer considered sensitive enough or specific enough to be used for the detection of CD, except in children younger than 18 months of age[201], although, iven if in association with IgA tTG, new-generation antibodies to deamidated gliadin peptides appear to be promising[202,203]. Antireticulin antibodies are also rarely measured, having been surpassed in use by EMA and anti-tTG antibodies.

The most sensitive and specific tests are the EmA and anti-tTG[204]; The IgA anti-tTG has 95% to 97% specificity and approximately 90% to 96% sensitivity. The serological gold standard is IgA EmA with its virtual 100% specificity, though a somewhat lower sensitivity than anti-tTG[119], and reports exist of lower sensitivity and specificity in the clinical practice setting[205-207]. Table VI summarizes characteristic of the celiac serological tests.

Table VI. *Operating characteristics of serological markers to detect the CD in adults[158].*

Serological test	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value
IgG AGA	57–78	71–87	0.2–0.9	0.4–0.9
IgA AGA	55–100	71–100	0.3–1.0	0.7–1.0
IgA EMA	86–100	98–100	0.98–1.0	0.8–0.95
IgA tTG	77–100	91–100	>0.9	>0.95
IgA tTG and EMA	98–100	98–100	>0.9	>0.95

Positive serological testing alone is not sufficient to confirm the diagnosis as there are a number of pitfalls to this assumption. First, falsely positive gliadin antibodies occur in a variety of other conditions such as inflammatory bowel disease[208]. Similarly, EMA is a qualitative test dependent upon the degree of immunoflourescence seen and positivity has been correlated with the degree of villous atrophy. In fact, CD[207] patients with lesser degrees of villous atrophy are less likely to have positive celiac serologies[207]. In fact, now we have learned that CD can be present with subtle, sometimes almost negligible, histological findings, and that it may be there even in the presence of a complete normal serology for any autoantibodies. In fact, it has become clear that in CD patients with only Marsh I type lesion (i.e., increased infiltration of intraepithelial lymphocytes, a nonspecific finding), the serology can often be negative[209,210], thus leading to underdiagnosis. Finally, also tTG has been found to be falsely positive in other autoimmune disease.

Children younger than 2 years of age lack EMA and tTG antibodies[211]; for this reason, serological testing in children younger than 5 years of age may be less reliable and requires additional study[199]. For these reasons, the diagnosis of CD cannot purely be based upon serological results. An additional consideration is antibody negative disease. CD could be associated with IgA deficiency[212] which may render both EMA and tTG negative as these antibodies are IgA-based. IgG-based tests are available but are still under investigation. Antibody negative CD can occur in individuals with a normal serum IgA level and may account for up to 9% of all cases of CD[213]. These individuals are often older and have more severe symptoms suggesting a diminished immunological response and therefore negative serology[213-215].

Although often positive test results can be supportive of a diagnosis, a small intestinal biopsy is the gold standard for the diagnosis of CD, and is used to confirm positive antibody test results[119,206,216].

The recognition of endoscopic signs of villous atrophy, such as scalloping of mucosal folds, absent or reduced duodenal folds, or a mosaic pattern of the mucosa (Appendix I, *Figure 2*), should prompt biopsy. As reported in paragraph 1.3 the spectrum of pathologic changes in CD ranges from near-normal villous architecture with a prominent intraepithelial lymphocytosis to total villous atrophy[73]. To avoid pitfalls in the pathological diagnosis, several parameters must be take into account. One of the commonest reasons for diagnostic uncertainty is that the individual has already commenced a GFD prior to the biopsy with subsequent histological improvement. It is essential that all individuals under the investigation CD remain on a gluten containing diet until a duodenal biopsy is performed. Furthermore, the number of biopsies taken is important as there appears to be histologically variable grades of villous atrophy within the same individual[217]. Current advice is that four or five biopsies should be taken from the second part of the duodenum (or more distally). In a recent work, the duodenal bulb frequently revealed villous atrophy (when placed in a

separate specimen container and marked for the attention of the gastrointestinal pathologist). There has been interest in adjuvant techniques for improving diagnostic yield such as the use of dye spraying with magnification endoscopy or confocal laser endomicroscopy[218], however these techniques require further training and as yet have not obviated the need for biopsy. When diagnostic uncertainty exists one strategy is to repeat biopsies following a period of gluten challenge (at least four slices of bread per day for 4–6 weeks) or consider jejunal biopsies via enteroscopy[219].

Recent evidence shows that the diagnostic accuracy of an intestinal biopsy can be greatly enhanced by measuring EMA and anti-tTG produced *in loco* by cultured duodenal mucosabipsies from patients with active CD or from patients in remission after in vitro stimulation with a peptic-tryptic digest of gliadin that usually contains the major immune stimulatory epitopes[220-223].

Other Authors[210] observed in a risk group of patients with potential or latent CD that 1% of these patients were positive for anti-tTG in serum and 6% were positive for anti-tTG in the cultures of duodenal biopsies, suggesting that local production of autoantibodies may precede subsequent histological damage. It is reasonable to add, however, that individuals with normal serology who only have Marsh I type lesion but positive duodenal anti-tTG need to be followed up clinically for a definitive diagnosis.Hence, the measurement of autoantibodies in the supernatant of the intestinal biopsy culture system was considered as an helpful tool to improve the diagnosis of CD in seronegative patients or patients with boubtful or borderline histological features. However, quality and size of the intestinal biopsy samples, their shor life span and standardization problems prohibited their wide use[224].

Another pitfall includes overinterpretation of villous atrophy in poorly oriented biopsy specimens and inadequate biopsy sampling in patients with patchy villous atrophy [225,226].

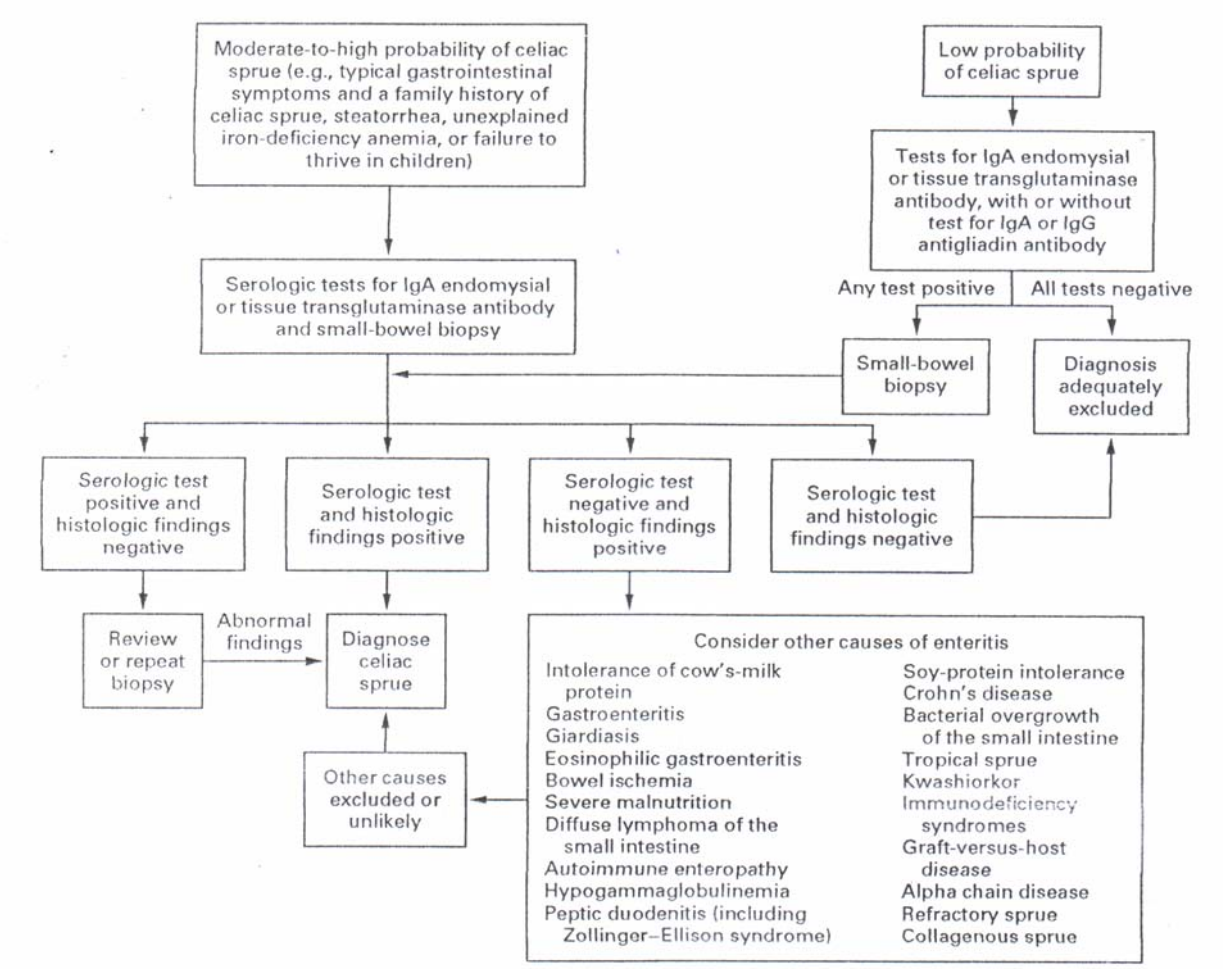
The histologic findings in CD are characteristic but not specific[227]; their presence permits a presumptive diagnosis of CD and initiation of a GFD. Indeed, CD is not the only cause of villous atrophy (Table VIII).

Table VIII. *Causes of villous atrophy other than CD[119].*

Giardiasis
Collagenous sprue
Common-variable immunodeficiency
Autoimmune enteropathy
Radiation enteritis
Whipple's disease
Tuberculosis
Tropical sprue
Eosinophilic gastroenteritis
Human immunodeficiency virus enteropathy
Intestinal lymphoma
Zollinger-Ellison syndrome
Crohn's disease
Intolerance of foods other than gluten (e.g., milk, soy, chicken, tuna)

Diagnosis depends on the finding of characteristic changes of intraepithelial lymphocytosis, crypt hyperplasia, and various degrees of reduced villous height, identified by intestinal biopsies, together with symptomatic and histologic improvement when gluten is withdrawn[119] (see paragraph 1.3).

Figure 7. *Diagnostic algorithm*[167].



As regards genetic assessment, as reported in paragraph 1.4.c the *HLA-DQ2* allele is identified in 90 to 95% of patients with CD, and *HLA DQ8* is identified in most of the remaining patients[228]. Because these alleles occur in 30 to 40% of the general population (with *HLA-DQ2* more common than *HLA-DQ8*), the absence of these alleles is important for its high negative predictive value[229]. Thus, the presence or absence of *HLA-DQ2* and *HLA-DQ8* is important for determining which family members should be screened with serologic testing and is useful for ruling out the disease in patients already on a gluten-free diet or for patients in whom the diagnosis is unclear.

Finallly, in the context of diagnostic approaches it is useful to underline that the detection of anti-tTG antibodies in human saliva is possible and may be useful to monitor adherence to GFD with a good correlation between saliva and serum titers (r=0.75, P=0.0001) [230].

Clinical-based evidence concludes that in certain high-risk symptomatic groups (eg, unexplained iron-deficiency anemia), individuals who initially have a negative serological test result may later develop a positive result (with repeat testing every few years) and subsequently have a biopsy compatible with CD[18]. Diagnosis of CD is often delayed for many years when disease progression in the small intestine is slow or mild. Investigators have noted a long duration of extraintestinal symptoms before diagnosis[231,232]. Studies report that symptoms are present for an average of 11 to 12 years [233] prior to diagnosis. The delay is thought to be due to physicians failing to diagnose the disease rather than patients not seeking medical care[233,234]. Absence of specific symptoms and the high variability in clinical presentation associated with CD have led to the misconception that it is a rare condition and physicians are not likely to suspect CD as a cause[17,235,236]. In national surveys[17,159,233], patients reported having received one or more different diagnoses before a definitive diagnosis of CD was made.

1.8. Treatment

Currently, the only scientifically proven treatment for CD is strict lifelong adherence to a GFD. All foods and medications containing gluten from wheat, rye, and barley, and their derivatives are eliminated, as even small quantities of gluten may be harmful. Complete removal of gluten from the diet in a patient with CD will result in symptomatic, serologic, and histologic remission in the majority of patients[17,237,238]. Growth and development in children returns to normal with adherence to the GFD and, in adults, many disease complications are avoided.

Green and colleagues[159] found that 70% of patients reported an improvement in symptoms within 2 weeks of initiating the GFD. With strict dietary control, antibody levels may revert to normal during 6 to 12 months of instituting the diet, complete histologic resolution may take up to 2 years[239]. In a small percentage of patients, it has been reported that small intestinal recovery and resolution of symptoms is incomplete[240].

Some patients have been found to suffer from refractory CD, a complicated form of CD in which patients may not respond entirely to the GFD[241,242]. Researchers have found that a non compliance rate of about 50% accounted for symptoms in patients with non-responsive CD[243].

Nutritional status of the newly diagnosed person with CD depends on the length of time that a person has lived with active but undiagnosed disease, extent of damage to the GT, and degree of malabsorption. At the time of diagnosis, some patients present with substantial weight loss, anemia, and evidence of overt vitamin/mineral deficiencies. A comprehensive nutritional assessment by a dietitian who is experienced in CD will determine the degree of malnutrition that exists[168,169,244,245]. Patients who present with nutrient deficiencies may require temporary or long-term nutrient supplementation with gluten-free vitamins, minerals and protein to correct deficiencies and replenish nutrient stores [168,169,244,245], although studies have not specifically looked at the efficacy of nutrient supplementation in the treatment of CD. Anemia may be treated with iron, folate, or vitamin B12, depending on the origin of the anemia. However, studies have shown that 78% and 94% of adults, respectively, recovered from anemia while being treated with a GFD alone[246]. Folate supplementation is recommended to ensure that the goal daily allowance is consumed until the damaged, functionally impaired villi heal in the absence of gluten in the diet[168,169,244,245].

Fat-soluble vitamin deficiencies (vitamins A, D, E, and K) are encountered in patients with classic malabsorption.

Recommended repletion dosages of fat-soluble vitamins are individually based; however, newly diagnosed patients may benefit from a water miscible form[168]. Calcium, phosphorus, and vitamin D deficiencies may occur due to malabsorption or a decreased intake of milk and dairy products in an effort to avoid lactose. In many cases, lactose intolerance resolves naturally with time on the GFD[167]. Given the considerable risk for bone disease in CD,

measurement of bone density, serum calcium, alkaline phosphatase, and parathyroid hormone levels is recommended at the time of diagnosis, and adequate dietary intake of calcium and vitamin D is essential. Gluten-free sources of calcium and vitamin D include most milk and dairy products (eg, calcium-fortified soy milk, rice milk, and juices); tofu; beans; canned salmon and sardines with bones; and cooked spinach, kale and broccoli. Some patients may not be able to meet the Recommended Daily Intake for calcium and vitamin D by diet alone[247], and supplements may be indicated[162].

Finally, since to conduct to a true GFD is increasingly challenging many laboratories are now working at different strategies to offer celiac patients more therapeutic options.

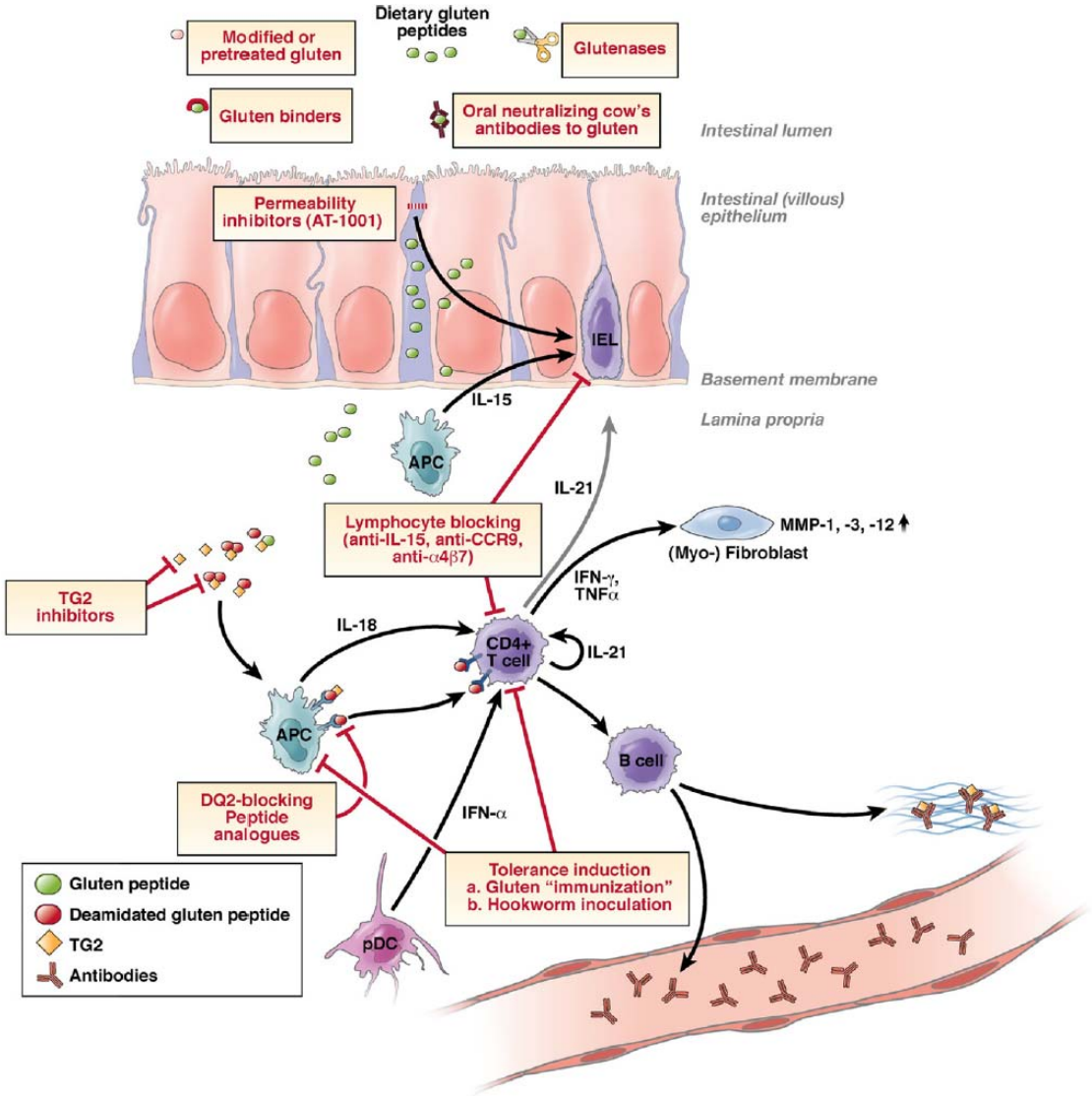


Figure 8. *Novel therapeutic approaches. Use of ancestral and/or modified wheat strains with lower immunogenicity. Intraluminal therapies that either bind or degrade ingested gluten peptides in the intestine (glutenases, gluten binders, neutralizing antibodies). Blocking the ZOT receptor with the octapeptide AT-1001 to decrease intestinal permeability is another option. Furthermore, because the deamidation of gluten peptides by TG2 and the subsequent presentation by HLA-DQ2/8 initiates the adaptive immune responses, TG2 inhibitors and DQ2 blocking peptides seem to be an attractive possibility to prevent inflammation. Another promising alternative, especially for patients with refractory celiac disease, is directly targeting the immune cells either by lymphocyte blocking (anti–IL-15, anti-CCR9, anti-α4β7) or tolerance induction*[117].

The novel therapeutic strategies that have been tested in *in vitro* or *in vivo* models of CD and approaches that may be promising in the near future are showed in Table IX and *Figure 8*. Therapies can be subdivided as to their intraluminal, epithelial, or subepithelial action[117].

Table IX. *Novel therapies for CD*[117].

Target	Drug/modification	State of development
Intraluminal therapies		
Wheat varieties	(Ancient) wheat variants with low immunogenicity	Preclinical, tested biopsy specimens and gliadin-reactive T-cell lines
	Genetically modified wheat variants or deletion lines of common wheat with lower immunogenecity	
Flour/dough	Pretreatment with lactobacilli	Clinical trial on 17 patients
	Transamidation of gliadin	Preclinical, tested on gliadin-reactive T-cell lines
Ingested gliadin peptides	Prolyl endopeptidases from Aspergillus niger	Phase 1 clinical trial (NCT00810654)
	Sphingomonas capsulate in combination with (EP)-B2 from germinating barley	Phase 1 clinical trial (NCT00626184)
	Intraluminal gliadin binding by polymers	Preclinical
	Gluten neutralizing cow's milk antibodies	Preclinical
Transepithelial uptake		
Epithelial tight junctions	ZOT receptor antagonist AT1001	Phase 2b clinical trial (NCT00889473)
Dampening of the adaptive immune response		
TG2	Transglutaminase inhibitors	Preclinical, tested ex vivo on biopsy specimens
	"Inhibitory" innate gluten peptides	Preclinical, tested on biopsy specimens and gliadin-reactive T-cell lines
HLA-DQ2	Blocking DQ2 analogues	Preclinical, tested on gliadin-reactive T-cell lines
Immune modulators		
	Hookworm infection	Phase 2 clinical trial (NCT00671138)
	Gluten "vaccination" (Nexvax2)	Phase 1–2 clinical trial (NCT00879749)
Biologicals (systemic T-cell or cytokine blockers)		
Small intestine homing T cells	CCR9 antagonists (Ccx282-B, CCX025)	Phase 2 clinical trial planned (NCT00540657)
Gut homing T cells	Anti-integrin $\alpha 4\beta 7$ (LDP-02)	Phase 2 clinical trial for Crohn's disease (NCT00655135)
Clonal IELs	Anti–IL-15 (AMG 714), Anti-Jak3 (CP-690-550)	Phase 2 clinical trial for rheumatoid arthritis (NCT00433875)
		Phase 2 clinical trial for rheumatoid arthritis, transplant rejection (NCT00550446, NCT00658359)
Clonal intestinal T cells	Autologous bone marrow transplantation	Clinical trial on patients with EATL
	Mesenchymal stem cell transplantation (prochymal)	Phase 2 clinical trial for Crohn's disease (NCT00294112)
Mucosal destruction in refractory celiac disease	Anti–tumor necrosis factor α, anti–IFN-γ (HuZAF)	Case reports in celiac disease
	Anti-CD52 (Alemtuzumab)	Phase 2 clinical trial for Crohn's disease (NCT00072943)
		Case reports in celiac disease

2. Celiac disease and oral cavity

As reported in paragraph 1.5.a among the atypical or extra-intestinal aspects of CD, literature has reported some affections interesting the oral cavity[248-250], mainly ALU[251-253] and DEDs[254-257], and secondarily unspecific forms of atrophic glossitis[258], oral variants of dermatitis herpetiformis[259]. Furthermore, Sjögren's syndrome (SS)[189,260] and OLP[261] occasionally reported in CD patients, must be considered as immuno-mediated diseases, sharing an immuno-mediated pathogenesis similar to CD[260,262].

2.1. Oral hard tissue lesions

2.1.a. Dental enamel defects

DEDs may be defined as a disturbances in hard tissue matrices and in their mineralization during odontogenesis, resulting in quantitative and/or qualitative enamel defects clinically obvious. The defect may be localize, affecting single or multiple teeth, or systematic affecting groups of teeth developing at the period of disturbance[263-265].

The reason for the presence of DEDs in celiac patients still remains not clear[257]. Hypocalcaemia due to malabsorption could be a possible cause, in agreement with the unifying hypothesis on mechanism of enamel hypoplasia development[266]. However, no differences in mean serum calcium concentration were found between celiac children with and without dental lesions[267], and celiac-type DEDs were also detected in healthy first-degree relatives of celiac patients having normal small-bowel mucosal architecture[268]. So, a gluten-induced, immune-mediated enamel damage should seem a more likely cause of DEDs in celiac patients. Consistently with this hypothesis, CD-associated dental changes were found significantly related to HLA antigen DR3[267-269]. Furthermore, a strong association between the same HLA allele and celiac-type DEDs were also demonstrated in healthy first-degree relatives of celiac patients[268]. Hypocalcaemia resulting from malabsorption could be nevertheless a contributing factor in inducing defective enamel formation[270].

Mineralization disturbances may be distinguished in hypoplasia and hypocalcification. Hypoplasia is defined as a quantitative defect of enamel involving the surface of the enamel with a reduced thickness of enamel. The defective enamel may occur as shallow or deep pits or small or large, wide or narrow grooves. Hypocalcification is defined as a qualitative defect of enamel identified visually as an abnormality in the translucency of enamel[271].

Furthermore, DEDs in CD, symmetrically and chronologically distributed, show specific and characteristic features as:

- symmetry of the lesions with involvement of different teeth;
- bilateral defects in both dental archs;

- chronological coherence: enamel defects follow chronology of dental amelogenesis, involving enamel surfaces that are forming when act pathogen agent[264,265].

The existence of an association between gastrointestinal disorders and dental enamel defects has been known since the turn of the century. In 1908 Black introduced the term "dental atrophy", which he used in reference to a deformity caused by a disease that interferes with correct nutrition[269].

In 1955, pronounced DEDs were described in a 7-year-old girl with clinical features of CD[272], and in 1956 seven patients with CD were examined, three of whom exhibited DEDs of the permanent dentition[273]. A case report of a 7.5-year-old girl with CD and hypoplastic permanent incisor teeth was presented by Pindborg[274]. In a Polish study of 18 children aged six to 17 years with a history of CD 20 hypoplastic teeth were found in eight children[275]. Thereafter, other cases of DEDs in subjects with CD were reported [273,276].

In a Swiss study, realized in 1980, of 252 patients with CD only nine were described as having mild to severe enamel hypoplasia[277]. Besides, the first controlled study available in English literature which investigated the presence of DEDs in CD patients reported no significant difference between celiac patients and healthy controls[251]. However, in 1986 Aine provided the strongest evidence that DEDs may be an extraintestinal manifestation of CD, reporting a 95.94% prevalence of DEDs of permanent teeth in 74 children with CD[278]. In this study, the celiac-type DEDs were defined as "systematic" (*i.e.* symmetrically and chronologically distributed in all four sections of dentition), and classified in four grades (Table X).

Table X. *Grading of dental enamel defects according to Aine (1986[254]).*

Grade	Dental enamel defect
Grade I	Defect in colour of enamel. Single or multiple cream, yellow or brown opacities with clearly definied or diffuse margins; a part or the entire surface of enamel is without glaze.
Grade II	Slight structural defects. Enamel surface rough, filled with horizontal grooves or shallow pits; light opacities and discoloration may be found; a part or the entire surface of enamel is without glaze.
Grade III	Evident structural defects. A part or the entire surface of enamel rough and filled with deep horizontal grooves which vary in width or have large vertical pits; large opacities of different colours or strong discolouration may be in combination.
Grade IV	Severe structural defects. The shape of the tooth changed: the tips of cusps are sharp-pointed and/or the incisal edges are unevenly thinned and rough; the thinning of the enamel material is easily detectable and the margins of the lesions are well defined; the lesion may be strongly discoloured.

Almost all the subsequent controlled studies related significantly higher prevalence values of DEDs in CD patients compared with non-celiac subjects, and confirmed that the celiac-type enamel defects are systematic, as differences for unspecific and unsystematic defects were not significant (Table XI). The different frequency likely depends on environmental, dietetic and, above all, genetic factors[279].

Table XI. *Prevalence of dental enamel defects in patients with CD.*

Reference	Dentition	Type of DED	Study group (patients with CD)	
			No. of patients	Prevalence of DED (%)
Malahias *et al.*, 2009	M, P [a]	systematic	67	51%
Avşar *et al.*, 2008	D, P, M	systematic	64	42.2%
Ortega Páez *et al.*, 2008	D	any	30	83.3%
	D	unspecific	30	10%
	D	systematic	30	73.3%
*Campisi et al., 2007**	D, M, P	systematic	197	23%
Procaccini et al., 2007	D, M, P	systematic	50	26%
Wierink *et al.*, 2007	D, M, P	any	53	55%
	D, M, P	unspecific	53	31%
	D, M, P	systematic	53	69%
Bucci *et al.*, 2006	D, M, P	systematic	70	20%
	D	systematic	17	5.88%
	M, P	systematic	53	24.53%
Farmakis *et al.*, 2005	D	opac., hypopl.[b]	13	92.30%
	P	opac., hypopl.	15	60%
Ciacci *et al.*, 2005	P	NR	360	16.11%
Priovolou *et al.*, 2004	M, P	any	18	83.33%
	M, P	systematic	18	44.44%
Rasmusson.and Eriksson, 2001	M, P	opac., hypopl.	40	50%
Lähteenoja *et al.*, 1998b	D, M, P	systematic	128	10.16%
Aguirre *et al.*, 1997	M, P	any	137	52.55%
	M, P	unspecific	137	14.59%
	M, P	systematic	137	37.95%
Rea *et al.*, 1997	D, M, P	systematic	45	24.44%
	D	systematic	15	13.33%
	M, P	systematic	30	30%
Ventura and Martelossi, 1997	M, P	systematic	603	32.33%
Martelossi *et al.*, 1996	M, P	systematic	90	53.33%
Bertoldi *et al.*, 1995	D, M, P	systematic	32	37.50%
Mariani *et al.*, 1994	D, M, P	systematic	82	28.04%
Ballinger *et al.*, 1994	P	systematic	42	9.52%
Petrecca *et al.*, 1994	M, P	systematic	29	75.86%
Aine *et al.*, 1992 [c]	P	any	30	100%
	P	unspecific	30	46.66%
	P	systematic	30	53.33%
Aine *et al.*, 1990	P	any	40	100%
	P	unspecific	40	17.50%

	P	systematic	40	82.50%
Balli *et al.*, 1988	D, M	NR[d]	49	34.69%
Prati *et al.*, 1987	D, M	NR	10	33.33%
Aine, 1986	P	systematic	74	95.94%
Andersson-Wenckert *et al.*, 1984	M, P	any	19	68.42%
	M, P	unspecific	19	15.78%
	M, P	systematic	19	52.63%
Shmerling *et al.*, 1980	NR	NR	252	3.57%
Romankiewicz-W. *et al.*, 1973	M, P	NR	18	44.44%
Miller, 1956 [g]	P	NR	7	42.85%
Hertz, 1955	D, M	NR	27	3.70%

[a] D=deciduous; M=mixed; P=permanent;; [b]opac.=opacities, hypopl.=hypoplasias;
[c] This study was performed on patients with dermatitis herpetiformis; [d]NR=not reported
* Data from this PhD study.

A few studies compared the prevalence of celiac-type DEDs in deciduous and mixed/permanent dentition. From all of these a higher prevalence of systematic enamel defects in mixed/permanent dentition emerges. In fact, a recent Italian study quoted 5.88% and 24.53% prevalence values of DEDs in celiac patients with respectively deciduous and mixed/permanent dentition[256]; in another paper[279] two out of 15 (13.33%) celiac patients had deciduous teeth with systematic enamel hypoplasia, compared with nine out of 30 (30%) celiacs with mixed/permanent dentition. Consistently with these figures, DEDs were detected in 1.68% and 29.56% of respectively deciduous and permanent teeth examined in celiac patients[267]. In a recent work[280], among CD patients, mixed dentition had significantly higher rate of DEDs than permanent dentition (90% *vs.* 34.0%, $p<0.0001$).

Taking into account the studies which have considered the systematic defects, the overall prevalence of CD-related DEDs in patients with mixed/permanent dentition ranges from 9.52% to 95.94% (with a mean value of 51.6%), whilst that for celiacs with deciduous teeth is 5.88% - 73.3%% (mean: 41,45%). The higher prevalence for permanent dentition is easily explainable considering that the development of crowns of permanent teeth occurs between the early months of life and the seventh year (*i.e.* after the introduction of gluten), while the formation of deciduous teeth mainly occurs *in utero*. But the presence of DEDs also in deciduous teeth supports the hypothesis that immuno-genetical factors are more likely involved in development of CD-related dental defects rather than nutritional deficiencies, or at least they represent the main cause.

The distribution of different grades of DEDs (according to the classification of Aine) shows that grade I and II defects are on the whole most common (Table XII).

Table XII. *Distribution of grades of CD-related dental enamel defects (Aine L, 1990)*[255].

Reference	Dentition	No. of celiacs with DEDs	No. of celiacs with grade I DEDs (%)	No. of celiacs with grade II DEDs (%)	No. of celiacs with grade III DEDs (%)	No. of celiacs with grade IV DEDs (%)
Ortega Páez *et al.*, 2008	D [a]	22	11 (50%)	4 (18.2%)	3 (13.6%)	4 (18.2%)
*Campisi et al., 2007**	D, M, P	46	40 (87%)	5 (11%)	0 (0%)	1 (2%)
Procaccini et al., 2007	D, M, P	13	10 (76.9%)	3 (23.1%)	0 (0%)	0 (0%)
Wierink et al.	D, M, P	20	3 (15%)	14 (70%)	2 (10%)	1 (5%)
Bucci *et al.*, 2006	D, M, P	14	7 (50%)	4 (28.57%)	2 (14.28%)	1 (7.14%)
Priovolou *et al.*, 2004	M, P	15	12 (80%)	1 (6.66%)	0 (0%)	2 (13.33%)
Aguirre *et al.*, 1997	M, P	52	32 (61.54%)	16 (30.77%)	3 (5.77%)	1 (1.92%)
Rea *et al.*, 1997	D, M, P	11	5 (45.45%)	4 (36.36%)	2 (18.18%)	0 (0%)
Martelossi *et al.*,1996	M, P	48	8 (16.66%)	22 (45.83%)	14 (29.17%)	4 (8.33%)
Petrecca *et al.*, 1994	M, P	22	10 (45.45%)	9 (40.90%)	2 (9.09%)	1 (4.54%)
Aine *et al.*, 1992 [b]	P	16	6 (37.50%)	10 (62.50%)	0 (0%)	0 (0%)
Aine *et al.*, 1990	P	33	15 (45.45%)	17 (51.51%)	1 (3.03%)	0 (0%)
Aine, 1986	P	71	10 (14.08%)	39 (54.93%)	14 (19.72%)	8 (11.27%)

[a] D = deciduous; M = mixed; P = permanent
[b] This study was performed on patients with dermatitis herpetiformis
* Data from this PhD study

Which factors determine the severity of DEDs are unclear at all. In fact, conflicting data become from the literature. It was reported that DEDs in adults are much less severe than in children, despite a similar prevalence[255]. In fact, no grade IV and only 1 case of grade III defects were found out of 40 adult celiac patients (with a mean age at diagnosis of CD equal to 34), whilst nearly 30% of children with CD had grade III or IV DEDs. This considerable difference could indicate that the adult patients had had a clinically mild or silent disease at childhood (with a consequent late diagnosis) and this determined slight dental lesions [255]. In support of this hypothesis, a correlation between the severity of CD clinical presentation and the grade of DEDs was also demonstrated in another study[254]. However, in another study a higher prevalence of enamel lesions in patients with atypical or asymptomatic CD was found[256].

Other studies considered the hypothesis of an association between late CD diagnosis and higher DEDs prevalence. Concordantly with the hypothesis that late diagnosis and prolonged gluten exposure relate to likelihood of DEDs, a few studies demonstrated that mean age at diagnosis of CD is significantly higher in patients with dental defects[281,282], although others failed to do so[256,267]. However, one of the latter papers[267], albeit found no

significant difference in age at diagnosis between celiac patients with and without DEDs, nevertheless noted in subjects with enamel lesions a correlation between age at diagnosis and the number of affected teeth. Furthermore, in a series of 360 adults with CD, no case of DEDs was described among patients with proved CD diagnosis in childhood and on a strict GFD since diagnosis, while DEDs were found in 18% of patients with early diagnosis but who were re-exposed to gluten, and in 26% of adults with newly diagnosed CD[283].

However, in a study on patients affected by dermatitis herpetiformis and without a history of symptoms suggestive of CD in childhood, no correlation between the degree of mucosal damage and the presence of DEDs was reported[284].

Concerning the location of CD-related DEDs, incisors are the teeth most frequently affected, followed by molars, canines and premolars[256,269 280,285,286]. This distribution should seem related to the chronology of development of the permanent dentition, as incisors and first molars are the first teeth that undergo calcification; the lesser involvement of teeth that calcify at a later stage could be explained considering that their calcification starts when CD has been possibly already diagnosed, and gluten removed from diet[269]. Instead, in non-celiac subjects dental defects seem to be equally distributed for all the teeth[256,269], with significant difference with respect to involvement of incisors as compared to CD patients[269].

In regard to the coronal distribution of DEDs, the incisal third resulted the most affected surface both in CD and control groups[280,285,286]; however, involvement of the incisal two thirds or of the complete crown were also observed in celiacs, while none of the control group showed this coronal involvement, and the difference resulted statistically significant[269].

2.1.b. Other hard oral tissue lesions

Several studies investigated the prevalence of dental caries in celiac patients. Balli et al. reported an higher dental caries prevalence in CD children[252]. In another recent study[287] the number of caries-free subjects in the control group was higher (38%) than in the CD group (17%).

Instead, other studies reported no difference in caries prevalence between celiac patients and control healthy subjects[251,257,264,277,288-291]. Rather surprisingly, others found significantly lower caries indexes (DMFT, DMFS, dmft and dmfs) in CD subjects as compared to control groups [251,269,291,292]. A possible explanation for these findings is that the need for a carefully controlled diet should make celiac patients more diet-conscious, so that they maybe have a low cariogenic diet [251,269,291].

Finally, dental age was studied in 49 children with CD{Ansaldi, 1989 #5930: delay in teeth eruption, estimated according to the dental standard of Schour and Massler[293], was noticed in 28.57 % of patients, this datum was subsequently confirmed by Prati *et al.*[288]. Similarly, in one-third of 38 children with CD examined in a Polish study the dental age was delayed in relation to calendar age[294]. Recently, in a Mediterranean sample, delayed eruption was observed by our research group (data from this thesis){Campisi, 2007 #13366} in 27% of the paediatric CD patients *vs* 7% of the controls (p<0.0001; OR=5.9).

2.2. Oral soft tissue lesions

2.2.a. Recurrent aphthous stomatitis

RAS (aphthae or canker sores) is one of the most common mucosal diseases known among humans, and the first use of the term "*aphthai*" in relation to disorders of the mouth is credited to the Greek philosopher Hippocrates[253].

RAS is a common condition which is characterized by multiple recurrent painful, round or ovoid ulcers with circumscribed margins, erythematous haloes, and yellow or grey floors, appearing first in childhood or adolescence[295].

As reported by Scully[296] the term "recurrent aphthous stomatitis" should be reserved to recurrent oral ulcer that present in patients without systemic diseases. Instead, ulcers that have a clinical appearance similar to RAS, but found in systemic disorders[297-302], if not present the typical clinical features or an onset in childhood are often termed ALU; but, generally these two terms are wrongly used indistinctly.

Depending upon the group examined, RAS may affect 5-60% of the general population, with a mean of 20%[303]. According to Stanley [304] RAS has three main clinical presentations: minor, major or herpetiform ulcerations. Patients may sometimes present with a mixed pattern of RAS, but this is relatively uncommon[301].

An association between oral ulcerations, diarrhoea and weight loss has been described for more than three centuries[305], but the first study about the association between CD and RAS date back to 1940[306]. Subsequently, many authors carried out several studies to confirm this association, but, the results are equivocal (Table XIII-XIV).

Table XIII. *Prevalence of CD in patients with RAS.*

Reference	Study group (patients with RAS)		
	No. of patients	Type of RAS	Prevalence of CD (%)
Shakeri et al., 2009	247	NR [a]	2.8%
Olszewska *et al.*, 2006	42	NR	4.7%
Aydemir *et al.*, 2004	41	NR	4.88%
Robinson and Porter, 2004	87	minor	0%
Nowak *et al.*, 2002	20	NR	5%
Jokinen *et al.*, 1998	27	NR	11.1%
Tavarela Veloso and Vaz Saleiro, 1987	24	NR	16%
Merchant *et al.*, 1986	100	minor	1%
Tyldesley, 1981	97	min., maj., her. [b]	6.2%
Ferguson *et al.*, 1980	50	minor	4%
Rose *et al.*, 1978	26	NR	3.8%
Ferguson *et al.*, 1976	33	NR	24.24%
Wray *et al.*, 1975	130	minor	3.8%
Ferguson *et al.*, 1975	35	NR	20%

[a] NR = not reported; [b] min. = minor; maj. = major; her. = herpetiform.

Table XIV. Prevalence of ALU in patients with CD.

Reference	Study group (patients with CD)		
	No. of patients	Prevalence of RAS (%)	Type of RAS
Malahias *et al.*, 2009	67	42.4%	NR [a]
*Campisi et al., 2008**	269	22.7%	Minor, major
*Campisi et al., 2007**	197	19%	NR
Procaccini *et al.*, 2007	50	36%	NR
Bucci *et al.*, 2006	72	33.33%	NR
Sood *et al.*, 2003	96	19.79%	NR
Sedghizadeh *et al.*, 2002	61	40.98%	NR
Lähteenoja *et al.*, 1998	128	3.12%	NR
Petrecca *et al.*, 1994	29	17.24%	NR
Corazza *et al.*, 1993	226	15.93%	NR
Meini *et al.*, 1993; Majorana *et al.*, 1992	113	17.69%	minor
Balli *et al.*, 1988	49	26.53%	NR
Biemond *et al.*, 1987	414	9.66%	NR
Andersson-Wenckert *et al.*, 1984	19	31.58%	NR

[a] NR = not reported
* Data from this PhD study

Oral ulceration akin to RAS can be a feature of GSE but only 5% of outpatients who initially present with RAS[307,308] have GSE.

The first study that investigated the prevalence of CD in patients affected by RAS reported very high values. Notably, Ferguson *et al.*[305] quoted a 24% prevalence of CD in 33 patients with RAS, and asserted the importance of performing jejunal biopsy in subjects with recurrent aphthae[309]. Subsequent studies[307,310,311] lessened this matter, albeit they reported CD prevalence values higher than those expected for the normal population, and thus supported the hypothesis of a correlation between CD and RAS. On the contrary, in 1986 only one of 100 patients with RAS had histological evidence of CD[312], and no CD-affected patient has been recently described among 87 subjects with RAS[313]. However, in the only controlled study that have searched for CD in patients with RAS, histological signs of CD have been demonstrated in two of 41 patients with RAS (4.8%), while none of the biopsies obtained from the 49 subjects of the control group revealed CD[314].

When papers examining the prevalence of RAS in CD patients are considered, the evidence of a correlation between the two conditions seems to be less robust. The prevalence of RAS in CD patients ranges from 3.12%[258] to 42.4%[280], a frequency similar to that reported in the general population (Table XIV).

A support for the hypothetical correlation between CD and RAS is provided by the observation that GFD may be effective in the management of RAS. In fact, several authors reported significant improvement, if not complete remission, of oral aphthae in most CD patients which had been placed on GFD [256,305,307,315-317], and reappearance of recurrences with the reintroduction of gluten[306,311,318,319]. However, Pastore *et al.*[250] referred that RAS does not always resolved after a strict GFD, this fact could be explained on the basis of the

multifactorial aetiopathogenesis of the RAS, although they reported a reduction of the RAS relapse.

Furthermore, patients with RAS and no histological evidence of CD showed a favourable response to GFD and a positive gluten challenge, suggesting the existence of a form of gluten-sensitive RAS[308]. On the other hand, a double-blind controlled study examined the effect of GFD in patients with RAS and without CD; four of the 11 subjects on GFD reported benefit as regards the oral aphthae, but no significant statistical difference was observed as compared with control subjects which underwent GFD supplemented by gluten given blind[320].

As regards the severity of aphtae, most papers assert that celiac patients with RAS suffer from minor aphthae[306,307,312,318,321]. However, cases of celiac patients with major or herpetiform RAS were also reported[311].

A possible explanation for the hypothetical correlation between CD and RAS is that oral aphthae in CD patients could be related to haematinic deficiencies (iron, folic acid or vitamin B12). In fact, even if in our recent article[303] we found haematological deficiencies in 56.2% of patients with recurrent aphthosis, it is generally accepted that about 20% of subjects with RAS may have a haematinic deficiency[322], and the serum levels of iron, folic acid and vitamin B12 are usually low in patients with untreated CD[323]. Consistently with this hypothesis, haemoglobin and serum folate levels were found significantly lower in patients affected by both RAS and CD compared to non-celiac patients with RAS[305,324]. Moreover, all the five cases of CD diagnosed in a series of 130 patients with RAS were deficient in iron, vitamin B12 or folic acid[321]. It has been suggested that the association between CD and RAS is related to the anemic status that frequently characterized an “atypical” celiac subjects[319]. In 1972, Lehner suggested that CD and RAS were the consequence of a similar immunological mechanisms[306,325],

Today, it is clear that the association between CD and RAS is not accidental, even if the causes of this link remains to be established.

The above-mentioned hypothesis, supported by the association between RAS and other immune-mediated gastrointestinal diseases, such as Chron’s disease and ulcerative colitis, is confirmed by a detection of an association between RAS and DR7 and the presence of an lymphocytic infiltrate of CD8+, CD4+ and NK into aphthous ulcerations[326,327]

Furthermore, Meini *et al.*[318] demonstrated an higher prevalence of haplotypes HLA-DRW10 and HLA-DQ1 in CD patients with RAS than CD patients without RAS, suggesting a genetic predisposition of CD patients to develop RAS.

An ultimate consideration regard “atypical” CD: RAS may represent the sole evident clinical sign of a asymptomatic malabsorption and must be consider as a possible proof of oligosymptomatic CD.

2.2.b. Non specific atrophic glossitis

A series of 128 patients with CD and 30 healthy controls was examined for oral mucosal lesions and symptoms in a Finnish study[258]. The tongue was most frequently affected, as 29.68% of CD patients referred soreness or burning sensation of the tongue, and the difference with the control subjects was statistically significant. Procaccini *et al*.[285] showed unspecific atrophic glossitis only in 4/50 patients with CD *vs* 1/50 control patients; whilst Campisi *et al*.[317] (data from this PhD study) found atrophic glossitis in 31/197 (16%) CD patients *vs* 1/413 controls (0.2%), whit all patients showing a resolution of the clinical picture after a period of GFD.

Pastore *et al*.[328] report the case of a man with erythema and atrophy of the dorsal tongue and without gastrointestinal complaints, whose serological tests for CD were positive; the small-intestine biopsy confirmed the diagnosis of CD, and GFD resulted in resolution of tongue lesion.

We found no more paper that supports a correlation between CD and glossitis. However, iron and vitamin B12 deficiencies are frequent in untreated CD patients[323], and it is well known that such deficiencies may cause atrophic glossitis, often accompanied by soreness or burning sensation. Furthermore, several reports describe cases of CD associated with Plummer-Vinson syndrome[329-335]. Plummer-Vinson syndrome (also referred as Paterson-Brown Kelly syndrome, or sideropenic dysphagia) is a rare condition that comprises the classical triad of dysphagia, iron-deficiency anaemia and esophageal webs; other clinical features may also be observed, among these atrophic glossitis[336].

2.3. Oral manifestations of dermatitis herpetiformis

Dermatitis herpetiformis (DH) is a pruritic bullous skin disease considered as a part of the spectrum of gluten-sensitive disorders. In fact, although patients with DH are unlikely to have gastrointestinal complaints, nearly all of them have some degree of small intestinal histopathologic changes identical to those of CD, and they share the same serologic antibody profile of celiacs[337]. Moreover, GFD is an effective therapy for DH, which confirms the role of an intestinal reaction to gluten in the pathogenesis of DH[338]. Typically, DH consists of symmetrical, erythematous, papulo-vesicular lesions that generally involve the extensor surfaces of the elbows, knees, buttocks and scalp. Owing to their intense itching, lesions often look as skin erosions secondary to scratching.

In a study on 15 patients with DH, oral lesions were found in 12 cases[339]. Oral involvement consisted of erithematous, pseudo-vesicular, purpuric and erosive lesions, but only in two cases the direct immunofluorescence examination revealed the typical granular deposits of IgA along the basement membrane, that is a *sine qua non* for the diagnosis of DH. Similarly, 57% of 27 patients with DH were found as having oral lesions, that included ulcerations, erythema and atrophies[340]; however, no direct immunofluorescence study was performed.

At the opposite, no oral mucosal involvement was described in a large review of 926 cases of DH[341], and only one case of small oral blisters was reported in a study on 149 DH patients [342]. Rather, in an old paper[343] the absence of oral lesions was referred as a diagnostic criterion for DH.

Actually, oral involvement in DH is at present considered very rare, and generally consists of vesicles and bullae that result in ulcers[344]. Reports that describe DH lesions in the oral cavity are sparse[259,345-348], and two of these[259,348] relate cases of oral manifestations appeared before the onset of the skin lesions.

2.4. Other oral manifestations

Other oral lesions described in celiac patients are oral T-cell lymphoma[349], carcinoma of the tongue and of the oesophagus[181,182,350,351] and oral mucosal melanosis[352]. Occurrence of T-cell lymphoma in the oral cavity is uncommon for celiac individuals, as CD-related lymphomas usually develop in the upper small intestine. The case of oral melanosis concerns a 32-year-old celiac woman, also affected by neuropathies and unspecified "dental irregularities"; GFD resulted in improvement of neurological findings, but not of oral hyperpigmentation. However, the association between CD and oral melanosis is likely occasional.

Interesting results have been recently obtained in a prospective, multivariate study on facial anthropometry of CD patients[353]. Face photographs of 120 celiac adults and 100 healthy controls were marked at predetermined reference points, and ratios of distances calculated by a computer program. CD patients diagnosed in adulthood and those diagnosed in childhood but who were not adherent to the GFD presented facial proportions significantly different as compared to both controls and celiac subjects with early diagnosis and on GFD since diagnosis. In particular, the former had a peculiar aspect of the face characterised by a larger forehead. Authors attributed this sign to an abnormal pattern of craniofacial growth due to nutritive and/or immunological factors, and proposed the "celiac face" as an extra-intestinal manifestation of CD.

2.5. Immunomediated disorders

SS[189,260] and OLP[261,354] have been reported in CD patients, but they should be considered expressively immuno-mediated diseases more than true oral manifestations of CD, since sharing pathogenesis and even the HLA DQ2 haplotype[260] as shown in SS[260]. As follows, it is reported the majority of the literature on this topic.

2.5.a. Sjögren's syndrome

Nutritional deficiencies in patients with SS were described since early 1950's[355], and in 1965 it was first reported a case of association between CD and SS[356]. Since then, several reports of patients affected by both the diseases have been published[189,357-362], as well as cases of SS occurring concomitantly with the gluten-related dermatitis herpetiformis[363,364].
In a study on 335 CD patients[365], 11 SS cases were found, with a prevalence significantly higher than those of healthy controls (3.28% *vs* 0.29%).
Five papers were identified that assessed the prevalence of CD in patients with SS (Table XV). One of these[366] is a large study on 5600 patients with various rheumatic complaints, 23 of whom were found affected by CD; SS resulted the most common rheumatic disease in celiacs, and CD prevalence in patients with SS was 9.52%. On the whole, the prevalence of CD in SS patients ranges from 2.53% to 14.70%[260,366-369]. Two out of these five studies were controlled[260,367]; both reported CD prevalence values in SS groups significantly higher than those of controls.
No significant difference concerning gastrointestinal symptoms was found between celiac and non-celiac patients with SS, suggesting that atypical or latent CD is more frequent in SS patients[368]. On the other hand, the mean age of SS patients with CD resulted significant lower than that of non-celiac SS patients, suggesting that CD should affect mostly younger SS patients[368].
The hypothetical link between CD and SS could be explained at least in part by a similar immunogenetic profile, since both diseases are associated with HLA DR3-DQ2 antigen[260,362,367]. Interestingly, GFD seems to be not effective on the sicca symptoms of patients with both SS and CD[260,366]. However, it has been suggested that the risk for autoimmune disorders in celiac patients is related to the age at diagnosis of CD and the duration of exposure to gluten [189], but this datum was not successively confirmed[370,371].
One study has recently examined oral mucosal and dental findings in patients with both CD and SS, and compared with those of patients with either CD or SS alone[362]. Prevalence of celiac-type DEDs was similar in CD and CD/SS groups, but significantly lower in subjects with SS alone. No difference was found as regards DMFT values in SS and CD/SS groups, whilst the caries index for the CD group resulted significantly lower; these findings suggest that the impaired salivary secretion should predispose to the development of dental caries

irrespective of the CD status. Oral mucosal lesions were found in 80% of SS, 65% of CD/SS and 40% of CD patients; the authors argued that the SS-related dryness of the mouth should affect oral mucosal health more than the inflammation associated with CD only[362].

Table XV. *Prevalence of CD in patients with SS*[248].

Reference	Study group (patients with SS)		Control group (subjects without SS)		CD diagnostic criteria	p value
	No. of patients	Prevalence of CD (%)	No. of subjects	Prevalence of CD (%)		
Szodoray *et al.*, 2004	111	4.50%	-	-	biopsy	-
Luft *et al.*, 2003	50	12%	50	4%	IgA anti-tTG	0.05
Calella *et al.*, 2002	79	2.53%	-	-	biopsy	-
Iltanen *et al.*, 1999	34	14.70%	28	0%	biopsy	NR [a]
Collin *et al.*, 1992	63	9.52%	-	-	biopsy	-

[a] NR = not reported

2.5.b. Oral lichen planus

In 1993, an unusual association of OLP and CD was reported[261]. This was the case of a 70-year-old male with a biopsy-proven erosive OLP; iron, folate and vitamin B12 deficiencies led to perform jejunal biopsy, that demonstrated CD. Surprisingly, GFD resulted in relief of OLP within six month. But the hypothesis of an association between CD and OLP was promptly refuted by Scully *et al.*[354], who one month later referred that they had investigated 103 patients with OLP and none had CD. So, they replied that OLP would seem only occasionally associated with CD.

In 1998, 39 consecutive patients with OLP were screened for CD; 12 were positive for IgA gliadin antibody test and two for endomysium antibody test, but only one had small intestinal signs of CD[372]. Furthermore, Ruiz Villaverde *et al.* described a case of a case of a 9-year-old female with erosive mucosal lichen planus associated to hyper IgE syndrome and CD[373].

2.6. Oral mucosa for initial diagnosis of CD or for gluten challenge

Although the small intestinal mucosa represents the main site of the gut involved in CD, other mucosal surfaces belonging to the gastrointestinal tract (GT) and to the gut-associated lymphoid tissue GALT can also be involved. In fact, modifications have also been found in the gastric[374,375], terminal ileal[376] and rectal mucosa[377-383]. Rectal mucosa, furthermore, has also been used for gluten challenges[381,384-386]. Finally, esophageal involvement has also been shown in patients with untreated CD[374,387]. All this demonstrates that gluten-driven T-cell activation is not restricted to the proximal part of the intestine but is present in the whole intestine, probably including the mouth.

In fact, as the first part of GT the oral cavity is the route for all materials ingested, including products made from cereals. It might be suspected that oral cavity would react to ingested gluten in the same way as the mucosa of the gut, showing oral changes when the disease is untreated and improvement in these changes when CD is treated with GFD.

In this context, differences in histological features of the oral mucosa of persons both with and without CD were demonstrated in a study that involved persons with GFD-treated CD, those with newly diagnosed CD, and healthy control individuals[258]. Moderate to severe lymphocytic inflammation was observed in 42.7% of the oral biopsy specimens from persons with treated CD, as compared with 10% of control individuals. Intra-epithelial T-cells were significantly increased in persons on GFD *vs* both untreated persons and healthy control individuals. The *lamina propria* of the persons with untreated CD had a significantly lower amount of mast cells as compared with that of both treated persons and control individuals.

These results were confirmed in another study by the same investigators[388], in which the T-cell count in the epithelium of treated persons was higher than that in untreated persons and control individuals, and the mastcell count in the lamina propria of treated persons was higher than that of untreated persons. Moreover, the mean amounts of CD3+, CD4+, and TCRgd+ cells were increased in the *lamina propria* of persons with CD and on GFD, compared with those in the other groups. The authors concluded that the oral mucosa cannot be used for the initial diagnosis of CD, since persons with untreated CD did not differ from healthy control individuals with regard to oral mucosal infiltration. In contrast, persons with treated CD showed significantly increased numbers of T-cell subsets in their oral mucosa, despite a strict adherence to a GFD; this finding was interpreted as a late immune response to minute amounts of gluten. Recently, another study[389,390](data from this PhD study) confirmed that no differences exist in $CD3^+$ count or B- and T-lymphocyte counts either in the epithelium or in the *lamina propria* of the oral mucosa in CD patients and in controls, although in the *lamina propria* of the CD patients there was a trend towards a higher number of $CD3^+$ and CD45RO lymphocytes.

The observation that, in the oral mucosa of persons with GFD-treated CD, an immunological 'memory' for gluten hypersensitivity is perpetuated prompted the authors to evaluate the reaction of the oral mucosa to a local gluten challenge[391,392]. A solution of an a-gliadin-related synthetic peptide was injected into the buccal submucosa of persons with GFD-treated CD and healthy control individuals[391]. The peptide significantly increased the T-cell counts in the lamina propria of persons with CD. The numbers of CD3+ and CD4+ cells were significantly higher after than before peptide challenge in persons with CD; the expression of the T-cell activation marker CD25 was also observed in the lamina propria of these persons after the challenge. The oral challenge induced no statistically significant difference in the cell counts of the control individuals. Importantly, neither the persons with CD nor the control individuals had any oral complaints or visible changes after the challenge; similarly, results of the serum EMA test remained negative after the challenge in all participants.

In another study, the same group performed the oral gluten challenge both at supramucosal (gliadin powder applied with an oral adhesive bandage) and submucosal (injection of gliadin solution) sites[392]. After a supramucosal challenge on the oral mucosa of persons with CD and on GFD, the number of intra-epithelial CD4+ and CD8+ T-cells increased in 67.6% and 73% of cases, respectively. Also, there was a significant increase in the number of CD4+ cells in the lamina propria of persons with CD. After a submucosal gliadin challenge, the mean numbers of total T-cells and CD4+ T-cells were significantly increased in the lamina propria of persons with CD. There were no differences in the mean cell counts of the healthy control individuals after submucosal challenge.

Based on these results, the authors reported a 73% sensitivity of the submucosal gliadin challenge in persons with treated CD, and a specificity of 80%; the positive predictive value was 93%, and the negative predictive value was 44%. Specificity and sensitivity of supramucosal challenge were not counted, since, in this study, control individuals were not tested for this kind of challenge. Mild pain or a burning sensation at the site of gliadin provocation was reported by some persons with CD, while others experienced mild mucosal changes (erythema, small blisters, swelling). Two persons with CD reported gastrointestinal complaints after the supramucosal challenge. The challenge did not have any influence on the serum EMA levels.

Recently, results from two groups of investigators have demonstrated that *in vitro* culture media of oral mucosa biopsies from persons with untreated CD can release IgA-class EMA and anti-tTG[393,394]. One of the groups (data from this PhD study)[393] found IgA EMA and antitTG in 53.6% and 57.2% of cultured oral biopsies, respectively. IgA EMA and anti-tTG assayed on culture media of duodenal biopsies were positive in all persons with CD. Interestingly, the results were identical when a 31-43 gliadin peptide was added to the culture systems. The sensitivity of the EMA test on oral mucosa media was 53.6%, the

specificity was 100%, and the diagnostic accuracy was 79%. For the anti-tTG test, sensitivity, specificity, and accuracy were 57.2%, 100%, and 79%, respectively. The second study[394] demonstrated IgA EMA and anti-tTG in all but one of the cultured oral mucosa biopsies from 16 persons with untreated CD. The single individual whose oral biopsy was negative for both IgA EMA and anti-tTG had a selective IgA deficiency.

Alla these findings are not surprising since the oral cavity is closely bound to the immunogenic defence systems. In fact, although the structure of the halthy oral mucosa differs from that of the bowel from an immunological point of view the lymphoepithelial structures of Waldeyer's pharyngeal ring in the posterior part of the oral cavity, including nasal-associated lymphoid tissues NALT constitute an important part of GALT. Although GALT is the largest and most important part of MALT [395], and after antigen-activation, proliferation and partial differentiation in GALT nad or MALT, memory B and T cells migrate to regional lymph nodes and secretory glands, including salivary glands where they can begin to produce sIgA[396], there is now compelling evidence that supports the compartmentalization of the mucosal immune system[397]. Therefore, activated immune cells seem to home preferentially to the sites where they were originally primed[398]. In this view, NALT and bronchus-associated lymphoid tissue (BALT) may be more important than GALT in inducing an immune response in the upper aerodigestive tract (*Figure 9*), including the oral cavity.

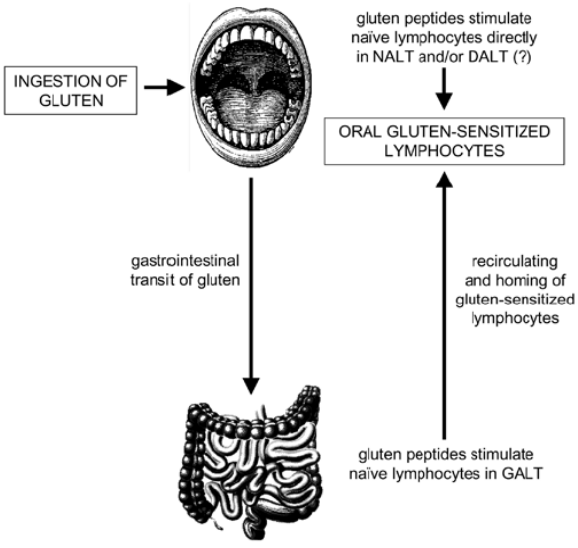


Figure 9. *NALT and BALT could have a role, independent from that of GALT, in inducing a gluten-related immune response in the oral cavity*[399].

Further studies should also investigate whether the oral gluten-sensitized lymphocytes do originate exclusively from homing of cells primed in GALT, or, rather, whether they could also be primed directly in the oral cavity (*Figure 9*). The latter would have important implications not only for diagnostics, but also for the understanding of CD in general.

The hypothesis that gluten could stimulate an immunological response directly in the oral cavity would have important implications for the understanding pathogenesis, diagnosis, and management of CD.

For example, the hypothesis of an induction of the gluten-related immune response even in the oral cavity should be taken into account in the development of new therapeutic strategies that aim to prevent the triggering of such immune reactions. Among these new non-dietary therapies under study, the most attractive seems to be the use of enzymes that digest immunogenic gluten peptides[400,401]; in the event that these peptides could already elicit an immune response in the oral cavity, enzymatic therapies could be ineffective or not fully effective, unless they are already active already in the mouth and not only in the stomach and/or in the small intestine.

Moreover, the hypothesis that a gluten-related immune response could already be induced in the oral cavity should be taken into account in the development of new non-dietary therapies that aim to prevent the triggering of such immune reactions only at the gastrointestinal level.

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CHAPTER II

PhD Studies

2.1. Aims

The aims of the PhD study were:

a) to examine the frequency of oral lesions in adults and children with CD and to assess their usefulness in making CD diagnosis;

b) to perform a screening for CD in patients with oral lesions potentially associated with CD, and sharing a common immune-mediate pathogenesis with CD, such as OLP and RAS;

c) to study the histomorphology of clinically healthy oral mucosa of untreated CD patients in order: (i) to assess the presence, quality (B/T lymphocytes) and intensity of lymphocytic infiltration and its relation to the severity of the intestinal histological damage; and (ii) to detect any other histological aspect which could be linked to CD;

d) to evaluate the ability of the oral mucosa of patients with CD to produce antibodies in an *in vitro* culture system.

2.2. Examination of the frequency of oral lesions in adults and children with CD and to assess their usefulness in making CD diagnosis

To achieve this objective two case-control studies were performed.

The *primary aim* of the **first study** was to assess the frequency of oral diseases, including soft and hard tissue lesions and dental delayed eruption in CD patients, in comparison with otherwise healthy controls; the *secondary objective* was to consider what contribution an oral clinical examination could give to help suspect a CD diagnosis.

The *objectives* of a **second case-control study** were to assess the prevalence of ALU in CD patients living in the Mediterranean area, and to evaluate the impact of a GFD.

2.2.a. Assessment of the frequency of oral diseases, including soft and hard tissue lesions and dental delayed eruption in CD patients and to consider what contribution an oral clinical examination could give to help suspect a CD diagnosis

Patients and Methods

The total study population of this prospective study consisted of 610 subjects; of these, 197 were CD patients, recruited at the time of diagnosis. They were consecutively enrolled, between January 2004 and June 2005: the adult patients (90 cases: 65 F, 25 M, age range 18-75 years, median 31) in two centers – Gastroenterology and Internal Medicine - of the University Hospital of Palermo and the children (107 cases: 59 F, 48 M, age range 2-17 years, median 9 years) in the Pediatric Gastroenterology Unit of the "Di Cristina Hospital" in Palermo.

CD patients aged under 2 years were excluded.

Four hundred and thirteen healthy subjects who were age/sex-matched (180 adults: 130 F, 50 M age range 19-77 years, median 32; 233 children: 120 F, 113 M, age range 2-17 years, median 8.5) and living in the same geographic area as the CD group, were enrolled as controls. Pediatric controls were recruited (by simple randomization) at a day nursery, and at Primary and Secondary Schools during a health prevention program for oral diseases; these subjects did not refer any diseases, had no family history of CD and showed normal growth (weight/height ratio between 25^{th} and 75^{th} centiles). Adult controls were recruited among otherwise healthy patients consecutively referred to the Dental Unit of the University of Palermo for third molar surgery; they were tested for serum anti-tTG antibodies and were negative.

CD diagnosis was based on the positivity of serum anti-tTG and/or anti-EMA antibodies, presence of clinical symptoms and evidence of intestinal histology damage on a gluten-containing diet, and the disappearance of the symptoms and normalization of serum anti-tTG and/or EMA on a GFD[1].

The clinical manifestations of CD were classified as "typical" when they included: chronic diarrhea, failure to thrive, anorexia, abdominal distension and muscle wasting; other clinical manifestations were considered "atypical"[2]. When CD diagnosis was made in subjects who were apparently asymptomatic, it was classified as "silent".

Immediately after CD diagnosis, the patients underwent intra-oral examination of the soft and hard tissues. All evaluations were performed independently by two oral medicine practitioners, including Dr. Domenico Compilato, who were trained in oral health survey, and then tested for concordance.

We focused on hard tissue lesions (i.e. DEDs), soft tissue lesions (e.g. presence of ALU, aspecific atrophic glossitis, geographic tongue) and clinical delay of dental eruption.

The enamel defects affecting deciduous and permanent teeth were graded 0 to IV according to Aine's classification[3] (see Chapter 1 paragraph 2.1.a). As regard ALU, we included recurrent ulcerative lesions clinically observed by the two dental practitioners during the intra-oral examination. However, as clinical evidence of the lesions is not always found because healing occurred before the oral investigation, but patients could have had a past history of aphthous ulcerations, we also considered ulcerative events, noted by parents or patients, or reported in hospital clinical records, with clinical features pathognomonic of RAS.

To evaluate delayed eruption in the pediatric patients we used the conventional eruption tables for the Caucasian Population[4] and we considered delayed eruption as when the teeth were not in arch after their normal age of eruption, with a range of ± 6 months.

In all individuals, dental hygiene was categorized into nominal variables using three values: 0 (poor), 1 (sufficient) and 2 (good)[5].

All CD patients with oral soft tissue lesions were re-evaluated one year after the beginning of GFD.

Finally, all the paediatric controls positive for oral hard and/or soft tissue lesions potentially associated to CD were tested for anti-tTG antibodies to exclude the disorder.

Informed consent was obtained for all participants in the study and the study was approved by the Ethics Committee of the University Hospital of Palermo.

Statistical analysis

Data were analyzed by means of SAS for Windows ver. 9.0, (SAS Institute Inc., Cary, NC, USA) and by means of Statistica 6.0 (Statsoft Inc, Tulsa, USA). To measure the association level, crude Odds Ratio (OR) and the 95% corresponding test-based Confidence Interval (CI) were calculated. Student's t test was used to calculate significant differences between cases and controls at baseline for normally distributed variables. The Mann Whitney U-test was used to calculate differences between not-normally distributed variables (e.g. Aine's scores).

The concordance rate of the oral evaluation between the observers was evaluated using Cohen's kappa statistic, measuring agreement beyond that expected by chance (expressed as a coefficient ranging from 0 to 1.00)[6].

The relationship between CD and other variables was analyzed within conditional multivariate frameworks.

A conditional logistic regression model was constructed stratified by age and dental hygiene, the same model permitted a stepwise selection procedure to obtain the most parsimonious model. The Maximum Likelihood Ratio (MLR) and Adjusted Odds Ratio (OR') were obtained using the iterative weighted least squares procedure. In all of the evaluations p values ≤0.05 were considered statistically significant.

Furthermore, in a blind manner three Artificial Neural Networks (ANNs) models were applied to the same data, as previously described[7]. Fully-connected multilayer feedforward networks were used. The learning rule employed was the well-known Back-error Propagation (BP) which adjusts the internal parameters of the networks over the repeated training cycles to reduce the overall error[8]. The networks were validated with a new set of data, different from the training ones. The performance measured by Mean Squared Error (MSE), Accuracy, Sensitivity and Specificity values as well as the Receiver Operating Characteristic (ROC) area, which can ascertain the degree of meaningful prediction [9,10] were calculated for the significant associations. In the present paper, all variables were selected as input ones, except for CD (the output variable) for ANNs system analysis.

Results

There was a substantial agreement (kappa = 0.85) between the observers for the main dental and oral lesions.

Table XVI summarizes the main findings of the oral examination in CD patients and controls.

Table XVI. *Oral examination findings in CD patients and controls, grouped by adult and pediatric age.*

	Adult individuals				**Pediatric individuals**			
	CD patients (*n* = 90)	**Controls (*n* = 180)**	**p-value**	**Unadjusted OR OR (95% CI)**	**CD patients (*n* = 107)**	**Controls (*n*= 233)**	**p-value**	**Unadjusted OR OR (CI 95%)**
Dental enamel defects								
no	69 (77%)	164 (92%)	0.001	3.12 (1.53 - 6.33)	82 (77%)	212 (91%)	0.0003	3.07 (1.63 -5.80)
yes	21 (23%)	16 (8%)			25 (23%)	21 (9%)		
Soft tissue lesions								
no	56 (62%)	177(97%)	<.0001	21.49	59 (55%)	229 (98%)	<0.0001	46.57 (16.14–134)
yes	34 (38%)	5 (3%)		(8.02-57.59)	48 (45%)	4 (2%)		
ALU								
no	71 (79%)	179 (99.5%)	<.0001	47.36	89 (83%)	231 (99%)	<0.0001	23.35 (5.31–102)
yes	19 (21%)	1 (0.5%)		(6.22-360.53)	18 (17%)	2 (1%)		

Forty-six out of 197 CD patients (23%) were found to have systematic and symmetric enamel defects *versus* a lower rate of 9% (37/413) in controls [p<0.0001; OR=3.510 (95%CI=2.135:5.770)]. The frequency of the enamel defects was very similar in the adult and pediatric CD patients. The severity of enamel defects in CD patients, evaluated according to Aine[3], was: grade I in 87%, grade II in 11%, and grade IV in 2%. (Appendix I, *Figures* 3, a-b).

Clinical delayed eruption was observed in 28 out of 107 pediatric CD patients (27%) *versus* 16 out of 233 (7%) controls [p<0.0001; OR=5 .932 (95%CI=3.407:10.330)].

The overall prevalence of oral soft tissue lesions was 42% (82/197) in CD patients and 9/413 in controls (2%) [*p*<0.0001; OR=22.257 (95%CI=13.828:35.824)]; frequency was similar in adult and pediatric CD. ALU was found in 37/197 (19%) CD patients *versus* 3/413 (1%) controls [*p*<0.0001; OR=18.9505 (95%CI=9.552:37.595)]. In CD patients ALU was directly observed during the medical visit in 34 cases and simply recalled by the parents in three cases. (Appendix I, *Figures* c-d).

The other soft tissue lesions detected were aspecific atrophic glossitis and geographic tongue. The first was found in 31/197 (16%) CD patients *versus* 1/413 (0.2%) controls [p<0.0001; OR=22.464 (95%CI=10.500:48.063)], while geographic tongue was noted in 14/197 (7%) CD patients *versus* 5 out of 413 (1%) controls [p<0.0001; OR=7.0326 (95%CI=2.650:18.666)]. CD patients showed a better dental hygiene status than controls [*p*<0.0001; OR=4.848 (95%CI=2.7027:8.695)].

None of paediatric controls with oral hard and/or soft tissue lesions present positive serological markers for CD.

As regards the clinical manifestations of CD, “typical” symptoms were more often observed in children (60/107 cases, 56%) than in adults (40/90 cases, 44%), whereas the frequency of silent cases was similar (6.6% in adults versus 7.4% in children). However, oral hard or soft tissue lesions were observed with an almost identical frequency in patients with “typical” and “atypical” CD symptoms. Furthermore, as among patients without any signs and symptoms potentially related to CD, as diagnosed during familial CD screening, there were also cases of oral soft tissue lesions, they should be considered as patients with “atypical” and not with “silent” CD. In CD patients, the frequency of the oral hard and/or soft tissue lesions did not differ according to the severity of intestinal mucosa damage or to the ideal body weight.

After a one-year follow-up, 33 out of 37 CD patients (89%) with ALU at diagnosis, referred that they had no longer suffered from ALU since beginning the GFD. The other 4 patients (11%) did not strictly adhere to GFD, as confirmed by persistently elevated serum anti-tTG antibodies, and did not report any improvement in ALU recurrence and number of ulcers per episode. Also atrophic glossitis disappeared in all the patients who adhered to GFD. As regards geographic tongue, no cases were present in the sample followed longitudinally.

Conditional multi-logistic analysis in the stepwise procedure selected Oral Mucosa Lesions and Dental Enamel Hypoplasia as the most meaningful variables in CD patients (Table XVII).

Table XVII. *Conditional multi-logistic analysis in the stepwise procedure. Characteristics and risk factors were stratified by age and oral care.*

	Odds Ratio	95% CI
Gender (Female vs Male)	1.980	1.253-3.130
Dental Enamel Hypoplasia	2.652	1.427-4.926
Oral soft tissue lesions	41.667	18.868-90.909

ANN methodology consistently proved that CD was the most meaningful variable related to soft tissue lesions in the present dataset, with MSE equal to 0.321. Performance indexes showed the following values: Accuracy= 83%; Sensitivity= 42%, Specificity= 98%. The ROC area was equal to 0.839.

On the basis of the 3% prevalence of CD recorded in our centers during the study period, and of the sensitivity and specificity showed by the ANN methodology, the positive and negative predictive values of the oral lesions were 39% and 99%, respectively. On the basis of the 1% CD prevalence in the general population, the positive and negative predictive values of the oral lesions in CD diagnosis were 17% and 99% respectively.

Discussion

Recent epidemiology data have shown a prevalence of CD approaching 1% in the general population[11-13]. However, there has been a noticeable change in the clinical presentation of CD, as almost 50% of the patients with newly diagnosed CD do not present with gastrointestinal symptoms[14,15] thus making diagnosis difficult. This is a paramount aspect as a GFD in CD patients is known to prevent many of the extra-intestinal symptoms, such as osteoporosis[16], recurrent abortions[17], and above all protects against the development of cancer[18]. Thus, to identify the greatest number of “atypical” or “silent” CD patients and prevent complications, clinicians must investigate “at-risk subjects”, e.g. those with chronic anemia[19,20], hyper-transaminasemia or hyperamylasemia of unknown origin[21,22], osteoporosis[16], autoimmune thyroid disorders[23]. Although the proximal part of the intestinal mucosa represents the main site of the gut involved in CD, it has been demostrates that gluten-driven T-cell activation is not restricted to the small intestine but is present in the whole GT. The mouth, the first part of the gastrointestinal system and a part of GALT, represents a site very easy to detect, in fact an oral examination could to give a useful diagnostic contribution since lesions of the hard[24,25] and soft tissues[26-31] have been reported in CD.

Ours is the largest uni-center study to have investigated the risk for CD patients of suffering from dental or oral mucosa lesions and showed the sensitivity and specificity of oral soft tissue lesions in suggesting a CD diagnosis.

As regards the hard tissues, we found systematic and symmetric DEDs in 23.3% of CD patients, with an odds ratio >3 in comparison with the controls. The enamel defects resulted in a dental deformity which can be easily recognized (see Appendix I, Figure 3, a-b), although low-grade lesions must also be accurately investigated. Other studies have reported a wide range of frequencies of enamel defects in CD patients[3,24,30-41] (see table XI on the paragraph 2.1.a) but our data are in agreement with other studies performed in Italy, and the differences in frequency probably depend on environmental, dietetic and, above all, genetic factors[34,42]. The same hypothesis could be made for the severity of enamel defects which appeared less severe in our study (87% of the patients had a grade I lesion) than in other studies[24] (see table XII on paragraph 2.1.a).

The etiopathogenesis of these defects in CD patients still remains unclear. Since the crowns of deciduous and permanent teeth develop from 4-5 months of intra-uterine life to the 7th year of life except for the third molar, nutritional, immunologic or genetic factors (association with the HLA DR3 allele) have been hypothesized as causing developmental defects in the enamel[33,43]. Hypocalcemia due to malabsorption during dental development has been considered to be implicated in enamel hypoplasia. Regarding nutritional dynamics, despite some doubts raised by Maki *et al.*[43], it has been hypothesized that a gluten-induced immunological process could occur between 6 months and 7 years in the enamel-producing organ, resulting in defective enamel formation. Finally, these dental anomalies have been found to be significantly related to HLA antigen DR3[33,36,43]. Furthermore, an Italian study[33], reporting a frequency of 28.8% in celiac patients versus 14.8% in controls, did not report any statistically significant differences in calcium concentrations, but a coincidence in 77.2% of CD and DEDs in DR3-positive subjects. Another finding on dental hard tissues was the significantly higher frequency of delayed eruption, observed in 27% of CD children. Only few papers in the literature have dealt with this issue[25,44-46] and they are in agreement with our data. The delayed dental eruption could be seen as a possible sign of malnutrition (such as is delayed puberty) and advises for serological CD screening.

However, the most important finding of the present study is related to the oral soft tissue investigation: in fact, mucosa lesions were found in 42% of CD patients with an odds ratio of 22 *versus* controls. Within this group of lesions, ALU was found in 37/197 (19%) CD patients with an odds ratio of 19 *versus* controls. ALU frequency in CD observed in our study is in agreement with those reported by other authors[47,48] (see table XIV on paragraph 2.2.a). It is also very interesting that almost all (89%) the CD patients with ALU no longer suffered after beginning GFD and the lack of healing in the remaining patients was probably linked to the lack of adherence to GFD. Consequently, ALU persistence in CD patients could cautiously be considered a marker of lack of GFD adherence. This hypothesis is supported by a study which reported that ALU and intestinal histological alterations relapsed after gluten challenge[49].

Except for a recent study[50] about the DEDs in CD children with deciduos dentition which showed that the presence in a child of systematic lesions had a sensitivity of 73.3% and specificity of 76.7% for the diagnosis of CD, and although several studies have reported the presence of oral mucosa lesions in CD, our study reports the first evaluation of the risk of such lesions in CD patients using univariate, multi-logistic regression, ANN sensitivity and specificity testing.

However, despite a good test accuracy, the presence of oral lesions showed a low positive predictive value of 16%, giving in the general population a 1% prevalence of CD.

In conclusion, our study showed a higher frequency of oral alterations in CD patients in comparison with healthy controls. However, the presence of these lesions had a low positive predictive value in CD diagnosis.

2.2.b. Assessment of the prevalence of ALU in CD patients living in the Mediterranean area, and to evaluate the impact of a GFD

Patients and methods

The study population consisted of 844 subjects living in the Mediterranean area. Local ethical committee approval was obtained.

The test group consisted of 269 CD patients (163 females, 106 males; range 3-17 years) consecutively enrolled, between 2004 and 2006, in two Italian centres: the Universities of Palermo and Ancona. Diagnosis of CD was based both on positive serology (IgA and/or IgG antibodies to htTG, together with positive histological evidence of villous atrophy with crypt hyperplasia and increase in intraepithelial lymphocytes, and the disappearance of symptoms and normalization of serum Ab-htTG after GFD.

The controls group were 575 otherwise healthy subjects age/gender-matched (343 females, 232 males; range 3-17 years) and living in the same geographic area as the CD group. Controls were recruited (by simple randomization) at a day nursery, and at Primary and Secondary Schools during an health prevention programme for oral diseases. These subjects were healthy, with no disease or previous positive medical history, or family history of CD and showed normal growth (weight/height ratio between 25th and 75th centiles).

Immediately after CD diagnosis, the patients in the test group underwent an intra-oral examination. Test and control patients were examined after obtaining informed consent in the presence of their parents who were questioned about a clinical history of ALU. In this study we included both ALU clinically observed and ulcers noted by parents or patients, or reported in hospital records.

According to Stanley[51] ALU has three main clinical presentations: minor, major or herpetiform ulcerations. The first are round ulcers less than 10 mm in diameter, while those major are clinically similar to the minor but they are larger than 10 mm in diameter[52,53].

All patients were examined in conventional dental chairs, using a dental operating light. All evaluations were performed independently by two dental practitiones, included Dr. Compilato, trained in an oral health survey and tested for concordance.

CD patients with ulcers or a history of ALU at the first visit were advised a GFD and re-evaluated one year after beginning this diet. At that second visit, adherence to GFD, serum anti-htTG and the frequency and severity of ALU were recorded.

Finally, all the controls positive for ALU were tested for serum coeliac antibodies to exclude CD.

Statistical analysis

Data were analyzed by means of StatView for Windows (SAS Inc v. 5.0.1, Cary, NC, USA). To measure the association level, OR and the 95% corresponding test-based CI were

calculated. Student's t-test was used to calculate significant differences between test and controls at baseline for ordinal variables. Chi-square test was used to assess statistical differences among categorical variables. In all of evaluations, *p*-values < 0.05 were considered statistically significant. The concordance rate of the oral evaluation between the observers was evaluated using Cohen's kappa statistic, as measuring agreement beyond that expected by chance (expressed as a coefficient ranging from 0 to 1.00)[6].

Results

No significant differences for age and gender (p>0.2 by Student's t-test and chi-square test, respectively) were detected between the test and control groups.

There was a substantial agreement (k=0.87) between the observers for ALU assessment.

ALU was found in 61/269 (22.7%) of CD patients *versus* 41/575 (7.1%) of the control group, showing a highly significant association of ALU with CD (*p*=<0.0001; chi-square=41.687; OR=4.3123; 95%CI =2.7664-6.722). The majority of ALU in both study groups were of the minor type (90.1% vs 87.8%; *p*>0.2). There were no differences in the number of episodes, rate of occurrence and duration (*p*>0.2).

None of forty-one control patients had positive serological markers for CD.

At the 1 year visit, there were 8 drop-outs (13.1%), meaning that 53 CD patients with ALU were interviewed and examined. Forty-six of these 53 patients adhering strictly to the GFD (87%): 33 (71.7%) had had no ALU, in 4 (8.7%) ulcers were reduced in frequency and severity, and in 9 (19.6%) there was no change in ALU history. In the other 7/53 patients who failed to strictly adhere to the GFD, six did not report any improvement in ALU, and Ab-htTG remained elevated. The difference in ALU history between test patients adhering verus those non adhering to the GFD was significant (*p*=0.0003; chi-square=13.101; OR=24.67; 95%CI =2.63:231.441).

Discussion

Atypical CD is characterized by non-specific intestinal complaints or by extra-intestinal manifestations including dental enamel defects, or mouth ulcers.

The frequency of the "true RAS" is debated, in fact, the literature reported different frequencies of RAS in the general young population: in some Brazilian studies it has been found in 0.9-1.57%[54,55]; Crivelli *et al.* described it in 10.87% of Argentine children[56], and Kleinman observed RAS only in 1.23% of 39206 United States schoolchildren[57,58]. ALU, also usually labelled RAS as well, have been reported in CD but with controversial frequencies[42,59,60]. In the present study, the prevalence of ALU was 22.7 % among 269 CD patients *versus* 7.1% in 575 individuals from general population living in the same area, with an odds ratio to suffer from ALU among the risk population of about 4.3; whilst other studies

shown a prevalence of 3.12-42.4% (Table XVIII). These differences may be explained by environmental, dietetic and, above all, genetic factors.

Table XVIII. *Prevalence of ALU in CD patients.*

Reference	Study group (patients with CD)		
	No. of patients	Prevalence of RAS (%)	Type of RAS
Malahias *et al.*, 2009	67	42.4%	NR [a]
Present study	269	22.7%	Minor, major
Campisi *et a.l*, 2007*	197	19%	NR
Procaccini *et al*, 2007	50	36%	NR
Bucci *et al.*, 2006	72	33.33%	NR
Sood *et al.*, 2003	96	19.79%	NR
Sedghizadeh *et al.*, 2002	61	40.98%	NR
Lähteenoja *et al.*, 1998b	128	3.12%	NR
Petrecca *et al.*, 1994	29	17.24%	NR
Corazza *et al.*, 1993	226	15.93%	NR
Meini *et al.*, 1993; Majorana *et al.*, 1992	113	17.69%	minor
Balli *et al.*, 1988	49	26.53%	NR
Biemond *et al.*, 1987	414	9.66%	NR
Andersson-Wenckert *et al.*, 1984	19	31.58%	NR

[a]NR= not reported

Thus, our data would support the hypothesis of a link between ALU and CD, but others have reported that only 5% of outpatients who initially present recurrent ulcers have evidence of CD[61,62]; consequently, in the absence of other systemic signs, screening for GSE among individuals with RAS has been generally considered fruitless[59]. However, on the basis of our findings of a very high percentage ALU in CD patients in a large population, we suggest to consider ALU, as proposed by Sedghizadeh[59], as a “risk indicator” for CD more than a “risk factor”; hence, the presence of oral aphthae should not be underestimated, but it should be considered a potential marker of underlying disease, above all if a patient has a positive personal and/or familiar clinical history. Consequently, serum CD markers (EMA and anti-tTG antibodies) should be suggested also for oligosymptomatic or asymptomatic subjects who showed ALU. However, it must be remembered the association of RAS with Behçet's disease and other inflammatory bowel diseases[63] and, consequently the association between ALU and CD cannot be considered specific. A further study has determined the positive predictive value of ALU in CD diagnosis[26].

A final point worthy of note is that in the CD patients suffering from ALU reported in our study a significant improvement in their ALU history 1-year after starting GFD, and conversely no improvement was observed in the CD who did not adhere to GFD.

2.3. Screening for CD in patients with oral lesions potentially associated with CD, and sharing a common immune-mediate pathogenesis with CD, such as OLP and RAS

The *aims* of a **first study** were: to assess the prevalence of CD in patients with OLP and the effects of GFD on this oral disease.

While in a **second study** tha *aim* was to assess the prevalence of CD in patients with RAS was performed.

2.3.a. ***Assessment of the prevalence of CD in patients with OLP and the effects of GFD on this oral disease***

Patients and methods

Twenty-three patients (21 F, 2 M; mean age: 58.7 years, range: 18-84 years) with OLP confirmed both clinically and histologically and under topical corticosteroid treatment were , were recruited during follow-up clinical examinations from the Oral Medicine Sector at the University of Palermo (Italy). Patients with lichenoid reactions to drug, amalgam fillings, topical allergens or any unknown reasons were excluded from this study. All patients were investigated for HCV-positivity and history of food intolerance and tested for serum coeliac autoantibodies. Serum samples were stored at 20 °C until analysis. They were analysed for EMA, tTGrh and tTGgp.

EMA IgA assay

Detection of EMA IgA was performed with indirect immunofluorescence using monkey oesophageal tissue as antigen (in-house assay). Tissue sections from marmoset monkey oesophagus were mounted on microscopic slides. Undiluted sera and sera diluted 1:25 with phosphate-buffered saline (PBS) was applied to slides, which were incubated for 30 min at room temperature. After washing with PBS the sections were covered with fluorescein conjugated rabbit-antihuman IgA (Dako, Copenhagen, Denmark) for 30 min, washed with PBS and examined by fluorescence microscopy. Positive sera were further diluted (1:5, 1:10, 1:25, 1:100, 1:400 and 1:1600). Sera positive in dilution 1:10 or more were defined as positive.

IgA tTG antibodies assay

The tTG antibody (IgA) tests were performed with two commercial ELISA-tests: (i) Celikey®, tTGrh, IgA antibody assay (Pharmacia Diagnostics, Freiburg, Germany). It was defined by the producer as positive when >8 U mL 1, negative when <5 U mL 1, borderline between 5 and 8 U mL 1, and (ii) ImmuLisa®, tTGgp with guinea-pig-derived tTG (IMMCO, Buffalo, NY, USA). The result was defined by the producer as positive when >25 U, negative when <20 U, borderline when 20 25 U. All sera were analysed using the same batch of tTGrh test. We used the upper borderline levels as a cut off for all the analyses as recommended by the producers. The intra-assay variation was measured as CV% (CV=coefficient of variation). The values for Celikey® were 6.0, 9.3 and 9.4 for low, medium and high values respectively. Corresponding values for ImmuLisa® were 3.9, 2.6 and 3.4 respectively. The inter-assay variation measured as CV% was 13.3 for Celikey® and 6.8 for ImmuLisa®, measured with a medium-high in-house control.

Finally, serum level of IgA, iron, folate and vitamin B12 were also assessed. All patients with positivity to EMA and/or anti-tTG autoantibodies underwent small intestinal biopsy to confirm CD, and in case of confirmed diagnosis were re-evaluated after 3 months of GFD.

Results

None of the patients showed HCV-positivity, history of foods intolerance and IgA deficiency. Only 2/23 (8.7%) female patients with atrophic/erosive OLP localized on buccal mucosa, associated to burning sensation, despite topical corticosteroid therapy, showed positive values of celiac antibodies associated to low serum levels of folate, vitamin B12 and iron. The small intestinal biopsy confirmed the diagnosis of CD. A GFD was started and the oral soreness and anaemia gradually improved over the next 3 month.

Discussion

OLP is a chronic inflammatory disorder affecting stratified squamous epithelia. The disease is relatively common, affecting approximately 1–2% of the population[64].

Histopathologically, OLP is characterized by the existence of an inflammatory infiltrate mainly composed of T cells in the papillary corion that acquired a characteristic band-like disposition and by a vacuolar degeneration of the epithelial basal stratum[65].

Although the aetiology of OLP is unknown, the histological features of the lesions suggest that the pathogenesis of this disease is immunomediate[65].

In fact, current data suggest that OLP is a T cell-mediated autoimmune disease in which auto-cytotoxic CD8+ T cells trigger apoptosis of oral epithelial cells[66].

Causative, associated or possibly worsening factors such as HCV and chronic liver diseases have also been involved.

The possible association between cutaneous-mucous LP and HCV infection is still controversial. A great number of studies carried out in Japan and southern Europe have identified high prevalence of anti-HCV antibodies in patients with OLP[67-70].

The HCV related OLP association is supported by the fact that HCV viral sequences have been found in the serum of patients with OLP, and HCV was shown to occasionally replicate in oral lichen planus tissue, possibly contributing to the pathogenesis of mucosal damage[69,71]. Moreover, recent data has shown that HCV-specific T cells can be found in the oral mucosa of patients with chronic hepatitis C and OLP[72].

OLP clinical relevance is owing to its high prevalence (up to 2%), to its association to a number of disorders, generally of autoimmune origin (myasthenia gravis, SS, ulcerative colitis, psoriasis, thymoma, lupus erithematosus, CD), and also to its potential for malignant transformation (about 0.4%).

About the association of OLP and other immuno-mediated diseases, although an high prevalence of OLP has been found in certain liver diseases such as primary biliary

cirrhosis[73], chronic active hepatitis[74] and cryptogenic liver diseases, it has been scarcely investigated with respect to gastrointestinal diseases (e.g. inflammatory bowel diseases and CD), although sharing immunomediate pathogenesis.

About OLP and CD, in 1993, an unusual association between these two conditions was reported[75]. This was the case of a 70-year-old male with a biopsy-proven erosive OLP; iron, folate and vitamin B12 deficiencies led to perform jejunal biopsy, that demonstrated CD. Surprisingly, GFD resulted in relief of OLP within six month. But the hypothesis of an association between CD and OLP was promptly refuted by Scully *et al.*[76], who one month later referred that they had investigated 103 patients with OLP and none had CD. So, they replied that OLP would seem only occasionally associated with CD. In 1998, 39 consecutive patients with OLP were screened for CD; 12 were positive for IgA AGA antibody test and two for EMA antibody test, but only one had small intestinal signs of CD[77]. Furthermore, Ruiz Villaverde *et al.* described a case of a case of a 9-year-old female with erosive mucosal lichen planus associated to hyper IgE syndrome and CD[78].

Data from our study, although obtained on a small sample size, allow to hypotize a potential association between CD and OLP; in fact in both cases the lesion is associated with a mucosal T-cell infiltrate. However, it is uncertain whether the oral lesions are a direct manifestation of GSE or due to the effect of malabsorption and of the consequently haematinic deficiencies on the rapidly dividing mucosal cells already predisposed to soreness by pre-existing OLP. In fact, a recent work[79] showed that 11 out 25 OLP patients suffered of low red cell folate levels.

Hence the resolution of the burning sensation after GFD, in our patients with OLP and refractory to local therapy could be justify the second hypothesis. Furthermore, a search for underlying nutritional deficiencies in patients with atrophic/erosive oral lesions should be important for the patients' general wll-being and in relieving oral soreness by nutritional means as opposed to palliative medications, which is often less successful.

Unlike its cutaneous form, OLP, and particularly the atrophic/erosive forms, is chronic with little likelihood of spontaneous resolution, so that the relief from eating a GFD in our cases was surprising.

2.3.b. ***Assessment of the prevalence of CD in patients with RAS***

Patients and methods

Over a period of 24 months, all patients with RAS who attended the the Section of Oral Medicine of the University Hospitsal of Palermo were asked to participate in a screening program for GSE.

Patients included in the study had at least three episodes of oral aphthae during the year; exclusion criteria were Behcet's disease, inflammatory bowel disease, systemic lupus erythematousis, tumors of oral cavity, Reiter syndrome and oral lesions due to drugs and radiation. Soft tissues examination was carried out with conventional dental chairs, artificial light, flat mirrors, monouse probe and sterile gauzes. We registered, lesions as RAS if they match one of these three conditions: clinically confirmed by physician, referred by patients themselves and reported by hospital clinical records.

The objectives of the study, as well as the possible necessity for a small bowel biopsy, were explained to the patients.

Of all patients with RAS only 25 (13 M, 12 F; mean age 42,4 ± 17,8 years; range: 7-77 years) agreed to participate in our study.

Written informed consent was obtained from each participant, and , after a gastrointestinal anamnesis, five-milliliter venous blood sample was obtained from each patient for serological investigation of GSE.

Patients were screened by IgA EMA, IgA anti-tTG (for procedure see the previous paragraph) and serum IgA level. Those with a positive serology underwent endoscopic biopsies of the duodenal mucosa.

Results

None of the patients referred gastrointestinal symptoms. Only 2 out of 25 (8%) RAS patients had a positive CD serology and the histological findings were compatible with GSE.

All of them showed a significant improvement of RAS episodes 1 months after beginning of GFD.

Discussion

RAS was found in 3.12%-42.4% of patients with CD[27,30], representing at least the fifth commonest presentations of CD[80-82] and often may be the sole presenting features of CD[27].

As RAS is frequently seen in CD patients, evaluation of individuals with this symptom may reveal the patients with undiagnosed CD.

Although the first association between RAS and GSE was proposed in 1975 by Ferguson *et al*.[83] when they found 20% of patients with RAS showed histological evidence of CD on jejunal biopsy, just recently the question has been raised by several Authors as to whether

RAS might be pathogenetically related to CD and whether GFD may induce improvement in RAS (Table XIX).

Table XIX. *Prevalence of CD in patients with RAS.*

Reference	Study group (patients with RAS)		
	No. of patients	Type of RAS	Prevalence of CD (%)
Present Study	25	NR [a]	8%
Shakeri *et al*., 2009	247	NR	2.8%
Olszewska *et al.*, 2006	42	NR	4.7%
Aydemir *et al.*, 2004	41	NR	4.88%
Robinson and Porter, 2004	87	minor	0%
Nowak *et al.*, 2002	20	NR	5%
Jokinen *et al.*, 1998	27	NR	11.1%
Tavarela Veloso and Vaz Saleiro, 1987	24	NR	16%
Merchant *et al.*, 1986	100	minor	1%
Tyldesley, 1981	97	min., maj., her. [b]	6.2%
Ferguson *et al.*, 1980	50	minor	4%
Rose *et al.*, 1978	26	NR	3.8%
Ferguson *et al.*, 1976	33	NR	24.24%
Wray *et al.*, 1975	130	minor	3.8%
Ferguson *et al.*, 1975	35	NR	20%

[a] NR = not reported; [b] min. = minor; maj. = major; her. = herpetiform.

Nevertheless, there is still considerable dispute concerning the actual prevalence of CD among patients with RAS, as different studies have reported different prevalence of CD in RAS patients[84-86]. As showed in table the prevalence of RAS in CD patients ranges between 0% to 24.24%.

On the other hand in recent years, some articles are published which expressed little or no significant etiological link between RAS and CD, and added that screening RAS patients for key serological markers of CD is of little clinical value[59,87]. Currently, there is no approved recommendation which can be used by clinicians to approach patients with RAS regarding celiac disease.

However, our findings although obtained from a limited sample population of patients with recurrent aphthosis, showed that a good percentage (8%) of patients with RAS had CD. If this results is compared with the 1% prevalence of CD in general population of Italy[88] this study suggest that evaluation of CD could be appropriate in patients with RAS.

The effect of GFD on remission of RAS is still uncertain, as dietary withdrawal of gluten occasionally results in significant benefit whereas some studies reported it ineffective [61,89]. In our study the 2 patients who started GFD showed an amelioration after only one month period of diet.

Many physicians may still consider the gastrointestinal signs and symptoms as a main manifestation of celiac patients whereas recent studies demonstrated that gastrointestinal

presentations may be absent in GSE patients especially in the beginning of the disease. In this study, none of our GSE patients had any gastrointestinal symptoms.

Therefore, gastrointestinal symptoms are sometimes absent in the setting of the disease and RAS could be the first or the sole presentation of GSE.

Our study has some limitations. We did not take duodenal biopsies from the patients who had negative serological tests. As reported in paragraph 1.6, It has been reported that the sensitivities of the serological tests are decreased in GSE patients with minor mucosal damages[90,91]. We cannot exclude the possibility of missing some GSE patients with negative serological tests and Marsh I/II mucosal lesions (e.g. seronegative GSE). However, a patient with negative serological test and duodenal mucosal lesion may suffer from other disorders like autoimmune enteropathy, giardiasis, common variable immunodeficiency, tropical sprue, peptic duodenitis, Crohn's disease etc. Including such patients (e.g. those with negative serological tests with duodenal mucosal damage) in the spectrum of GSE could increase the rate of false positive results; unless symptomatic and histological improvements are confirmed by GFD. Therefore, in the epidemiological studies, a positive result from a highly specific serological test (e.g. EMA, or tTG) together with any degree of duodenal mucosal lesion provide reasonable criteria for identifying patients with GSE.

Although our study was conducted on a small sample size, we can conclude that a subset of RAS patients could suffer from GSE. GSE should be considered in RAS patients; unresponsiveness to conventional anti-aphthae treatment could be an additional risk indicator. Implementation of GFD may prevent the complications of GSE and effectively treat RAS.

2.4. Study of the histomorphology of clinically healthy oral mucosa of untreated CD patients

The *aims* of this study were: (i) to assess the presence, quality (B/T lymphocytes) and intensity of lymphocytic infiltration and its relation to the severity of the intestinal histological damage; and (ii) to detect any other histological aspect which could be linked to CD.

Patients and methods

The test group consisted of 21 newly diagnosed, and untreated CD patients (15 females and 6 males, median age 36 ± 14 years; range 13–68 years) consecutively enrolled, between June 2004 and May 2005, in 3 centers: the adults in two centers: Gastroenterology and Internal Medicine of the University Hospital of Palermo; while the children in the Pediatric Gastroenterology Unit of the 'Di Cristina Hospital' in Palermo.

During the same period, 14 subjects (10 females and 4 males, median age 39 ± 15 years; range 12–66 years), matched for age and gender, were consecutively recruited from patients with symptoms compatible with CD diagnosis (weight loss, failure to thrive, anemia, chronic diarrhea, abdominal pain, dyspepsia, gastro-esophageal reflux and abdominal meteorism), but with negative serum assays for CD diagnosis. All underwent esophago-gastro-duodenoscopy (EGDS) with duodenal histological examination. All showed normal histology of the intestinal mucosa and were considered control subjects.

CD diagnosis was based on positive serology (IgA and/or IgG anti-tTG, IgA and/or IgG EMA) together with positive histological evidence of villous atrophy with crypt hyperplasia and increase in intraepithelial lymphocytes, and the disappearance of symptoms and normalization of serum anti-tTG and/or EMA after a GFD was imposed[1].

Test and control patients were recruited after a careful clinical examination of the entire oral cavity, performed at the Oral Medicine Unit of the University Hospital of Palermo, and subjects showing soft tissue lesions (e.g., ALU, non-specific atrophic glossitis, mucosal erythema) were excluded from this study.

Informed consent was obtained from all of the patients involved in the study or from their parents in the case of pediatric patients.

Intestinal and oral biopsies, histological and immunohistochemical evaluation.

Following general anesthesia (in children) or conscious sedation with meperidine/pethidine 1 mg/kg i.v. and midazolam 0.05–0.1 mg/kg i.v. (in adults), EGDS were performed with standard pediatric or adult videocolonoscopes (Olympus, Milan, Italy). At least four duodenal biopsy samples were obtained from each patient.

Both 21 untreated CD patients and 14 controls underwent oral mucosal biopsy. Biopsy specimens were taken at the same time as the EGDS in the children and under local

anesthesia (Xylocaine® 2%, adrenaline 1:100.000) in adults. The specimens were always taken by Dott. Domenico Compilato from a clinically healthy buccal mucosa adjacent to the right second molar tooth region, beneath the occlusal line. The biopsies were performed by punching (6 mm in diameter punch biopsy) or by incision with cold knife.

The intestinal and oral biopsy specimens were immediately fixed in 10% neutral-buffered formalin for 2 h, washed in water for 1 h, dehydrated in graded ethanol (60%, 80%, 90%, 95% and, 100%) and after permeation in xylene, embedded in paraffin using the standard procedures. The formalin-fixed, paraffin-embedded samples were cut into 5-μm-thick sections on a microtome with a disposable blade. The sections of the intestinal mucosa were stained with hematoxylin and eosin (H&E) for standard evaluation. From each specimen of the oral mucosa, three sections were obtained: one conventionally stained with H&E and the other two treated with 4 KB5 monoclonal antibodies against- CD45RA and with UCHL1 monoclonal antibodies against-CD45RO (Dako, Milan, Italy) to evaluate lymphocyte infiltrates B and T, respectively.

Intestinal histological specimens were defined compatible with the clinical diagnosis of CD when there was both intraepithelial lymphocytosis (at least 40 lymphocytes/100 enterocytes)[92] and a villous height to crypt depth ratio (V/C) <2.5. Furthermore, based on this ratio, a CD histological grading was defined: Grade 1 = V/C 2–2.5; Grade 2 = V/C 1–2; Grade 3 = V/C 1–0.5; and Grade 4 V/C < 0.5[93,94].

All oral biopsy slides were examined blindly by Prof. Emiliano Maresi. CD3+ IELs were examined using mAb Leu-4 (Becton Dickinson, San Jose, CA). B lymphocytes were detected in paraffin-embedded sections using monoclonal antibodies to CD20 (Dako a/s, Glostrup, Denmark) and T lymphocytes with monoclonal antibodies to CD45RO (Dako a/s).

The densities of $CD3^+$, $CD20^+$ and $CD45RO^+$ cells were light microscopically counted in the epithelium and in the *lamina propria* using an ocular graticule of 0.10 x 0.10 mm at x400 magnification. Ten fields were counted in the buccal epithelium and *lamina propria* immediately below the basement membrane. The results were given as cells/mm^2. The intensity of the *lamina propria* lymphocytic infiltrate in the H&E stainings was arbitrarily graded as none (0), low (1), moderate (2) or severe (3).

Furthermore, the presence of a spongiotic tissue reaction was assessed. Spongiotic reaction was defined as the presence of intraepidermal and intercellular oedema and labeled “spongiosis’”.

Statistical analysis

Data were analyzed by means of STATVIEW for Windows (SAS Inc v. 5.0.1, Cary, NC, USA). To measure the association level, OR and the 95% corresponding test-based CI were calculated. Chi-square test was used to assess statistical differences between categorical variables. The Mann–Whitney U-test and Spearman rank correlation coefficient

(Spearman's rho) were used to calculate differences between non-parametric continuous variables. Student's t test was used for parametric continuous variables. In all of the evaluations, P-values ≤0.05 were considered statistically significant.

Results

Table XX shows the lymphocyte (B and T) counts in the oral mucosa of the 21 CD patients and of the 14 controls. No differences were observed in $CD3^+$ count or B- and T-lymphocyte counts either in the epithelium or in the *lamina propria* of the oral mucosa in CD patients and in controls, although in the *lamina propria* of the CD patients there was a trend towards a higher number of $CD3^+$ and CD45RO lymphocytes.

TABLE XX. *Number of T- and B-lymphocytes in the oral epithelium and in the lamina propria of 21 untreated CD patients and of 14 controls (IELs were counted as cells/mm^2; lamina propria lymphocytes as cells x 100 / mm^2). Data are given as mean ± SD.*

	CD Patients	Controls
IELs		
CD3+	152 ± 121	168 ± 110
B-Lymphocytes	43 ± 42	38 ± 45
T-Lymphocytes	121 ± 102	131 ± 98
Lamina Propria		
CD3+	5.1 + 4.7	3.1 ± 3.8
B-Lymphocytes	0.8 + 0.9	0.6 + 0.7
T-Lymphocytes	4.5 + 2.9	3.8 + 3.9

Furthermore, there was no relationship between the intensity of lymphocytic infiltration in the oral mucosa and severity of intestinal mucosal damage, evaluated as villi/crypts ratio (r = 1.03, *p*= 0.8) (Table XXI).

TABLE XXI. *Relationship between the intensity of lamina propria lymphocyte infiltration in the oral mucosa and severity of intestinal mucosa damage.*

	Intestinal CD histological grading				*Number of CD patients*
	Grade 1	***Grade 2***	***Grade 3***	***Grade 4***	
Grading of oral lymphocytic infiltrate					
none	0	0	1	5	6
low	0	2	3	9	14
moderate	0	0	0	0	0
intense	0	0	0	1	1

A very frequent histomorphological finding was spongiosis of the oral mucosal epithelium, observed in 19⁄21 (90%) of CD patients and only in 4⁄14 (29%) of controls [p= 0.0002; x^2 = 14.29; OR = 23.75 (95%CI = 3.69:152.9)]. This spongiotic reaction was characterized by the presence of intraepidermal and intercellular oedema; intercellular spaces were widened with an elongation of the intercellular bridges (Appendix I, *Figures 4-5*). The foci of spongiosis were also characterized by a mild lymphocytic infiltrate, but not by the presence of eosinophils or neutrophils. These lesions were exclusively microscopic without any corresponding identifiable vesicles on the oral mucosal surface.

Discussion

Although histological and clinical features of the small intestinal mucosa in CD are well known, the involvement of the other mucosal surfaces belonging to the GT and to the GALT has been neglected. As reported in Chapter I (paragraph 2.6), it has been demonstrated that in CD patients, gluten-driven T-cell activation is not restricted to the proximal part of the intestine, but is present in the whole intestine. In fact, the dissemination of the immune effector cells involves very distant sites of the GALT including the oral and rectal mucosa[95], the two extremities of the GT.

Histological changes have already been shown in the gastric[96,97], terminal ileal[98], rectal mucosa[99-101], and esophageal mucosa[96] of CD patients.

Although it is widely reported that in CD patients, the mouth can also be affected by several clinical manifestations such as ALU[42,49,59,102], DEDs[24,29,103,104] and non-specific atrophic glossitis[27], data about histological features of clinically healthy oral mucosa in patients with CD are few and often contrasting[27,105-107].

The healthy mucosa of the mouth is quite different from that of the rest of the GT tract; the oral mucosa of the lips, buccal, and floor areas are covered by a non-keratinized stratified epithelium, while the lining of the stomach and small and large intestine consists of a simple epithelium composed of only a single layer of cells[108]. Chapter I (paragraph 2.6), although the structure of the oral mucosa differs from that of the bowel from an immunological point of view, the mouth is situated at the beginning of the gastrointestinal system and, as part of it, is considered to belong to the GALT. Consequently, after antigen activation, proliferation and partial differentiation in the GALT, memory B cells and T cells migrate to other parts of the GALT, including the oral mucosa lymphoid tissues[95]. There, these cells, in sensitized CD patients, may easily react to gluten and its peptides; in fact, more recent findings have suggested that the oral mucosa of CD patients could be useful to evaluate response to a gluten-challenge[106].

Based on these considerations, we expected the healthy oral mucosa of untreated CD patients to behave in a similar way to the small bowel mucosa. However, our results did not confirm our initial hypothesis and we did not find any significant presence of B and T

lymphocytes infiltrate or any relationship with severity of CD in the oral mucosa of our study group.

These data are in agreement with a study performed in Finland in which untreated CD patients did not differ from controls in terms of T cell number in the buccal mucosa[27,105]. The behavior of the oral mucosa of our patients with untreated CD did not mirror that of the small intestinal mucosa in which an increase of T cells generally occurs and the density of these cells decreases on a GFD. An explanation of this result might be that in patients with untreated CD, a gluten-containing diet induces a sequestration of lymphocytes to the intestinal mucosa and hence a "concentration" of the inflammatory cells with greater mucosal damage at this site[108,109]. This may result in a low intensity of lymphocytic infiltrate in other mucosal tissues belonging to the GALT, including the oral mucosa[27,110].

The novel finding of our research is the detection of 'spongiosis' in the biopsy samples of the clinically healthy oral mucosa of untreated CD patients. We found epithelial spongiosis in almost all the study group (90%) with a crude OR of about 23.

In general, spongiotic tissue is characterized by the presence of intracellular and intercellular oedema. Indeed, spongiosis is considered only a histopathological concept and not as a clinical entity, although several diseases present such a tissue reaction[111].

Spongiotic reaction has been found in several skin lesions[112-116] and in some oral diseases, such as oral melanoacanthosis (melanoacanthoma)[117], oral psoriasis[118], allergic contact stomatitis[119], plasma cell gingivitis, intra-oral fixed drug eruption, leukoedema and white sponge nevus[120]. In most of these oral lesions, the pathogenetic mechanism involved in the collection of the intraepithelial fluid is not clear and remains to be elucidated: spongiosis could be caused by extravasations of fluids from blood vessels located in the lamina propria or by the presence of an osmotic gradient developed towards the epithelium, drawing fluid into it subsequent to various immunological reactions[111].

Spongiosis is also a histopathological feature found in the epithelium at the margin of an aphthous ulcer[120]; moreover, the pre-ulcerative stage in the pathogenesis of RAS is characterized by oedema of the epithelium and keratinocyte vacuolization[121], and therefore by spongiosis.

However, as several conditions, like the presence of mucopolysaccharidic materials may simulate spongiosis, we demonstrate that this histomorphologic feature is not associated with the accumulation of PAS-positive substances (e.g., acid mucopolysaccharides) within the cytoplasm of the keratinocytes with spongiotic aspect (Appendix I, *Figure 6*).

We underline that our finding of massive spongiosis was found in the otherwise healthy oral epithelium of CD patients. Noteworthy, this finding may support the hypothesis that spongiosis represents histological evidence or a key of the well-known higher susceptibility of these patients to suffering from ALU. The continuous contact of the gluten-containing foods with the oral mucosa could provoke epithelial spongiotic changes, characterized first

by intracellular oedema and then by intercellular oedema with elongation of the intercellular bridges. The increase of intercellular oedema leads to the formation of intraepithelial micro-vesicles and thereafter breaking off to form ulcer. According to this theory, patients not adhering to GFD would be more likely to suffer from RAS, as actually verified by our research group and reported in the previous paragraph[102,103].

The lack of lymphocytes infiltration of the oral mucosa cannot exclude that other inflammatory mechanisms, cells and mediators play a role in determining an inflammatory condition that determines the onset of spongiosis.

A further datum comes from a recent study[122] in which authors showed that the clinically healthy oral mucosa of patients who underwent allogeneic hematopoietic stem cell transplantation presents the same histological changes as those observed in biopsy specimens of patients with clinical evidence of oral chronic GVHD; in this case, a basal cell hydropic degeneration resembling the spongiosis reported by us was observed.

This latter study, as well as ours, proposes that oral biopsy could represent an important and useful diagnostic method for predicting the clinical onset of oral lesions in patients with some systemic diseases, such as CD or c-GVHD, especially if we consider that the oral cavity represents a site with less invasiveness and with an easy access.

In conclusion, our findings confirm that the oral mucosa of untreated CD cannot be considered a counterpart of the jejunal mucosa, at least in terms of B and T lymphocyte infiltrate.

The finding of oral mucosal spongiosis, to the best of our knowledge, is the first reported for CD; its meaning is not definitively explained, but it could provide the wellknown susceptibility of CD patients to RAS onset. In these patients with untreated CD, oral mucosal spongiosis may be related to histological phenomena induced by gliadin ingestion. Hence, future aims could be to demonstrate whether oral spongiosis is a reliable sign of naÏve CD, and whether it should be used as a marker of untreated CD, an indicator of non-adherence to GFD or a condition present despite strict adherence to GFD.

2.5. Evaluation of the ability of the oral mucosa of patients with CD to produce antibodies in an in vitro culture system

Patients and methods

Patients with new CD diagnosis observed in three centers (one for pediatric - Pediatric Gastroenterology Unit of the 'Di Cristina Hospital' in Palermo - and two for adult patients Gastroenterology and Internal Medicine of the University Hospital of Palermo), between June 2004 and May 2005, were randomly invited to enter the study. Out of a total of 105 new diagnoses, 51 patients were asked to participate and 28 subjects accepted and were recruited. Of these, 15 were adults (10 F, 5 M, median age 39 years, range 19-73 yrs) and 13 children (10 F, 3 M, median age 7 years, range 1-16 years). The CD clinical presentation was typical (malabsorption syndrome) in 14 patients, atypical (absence of intestinal symptoms) in 12 and silent in 2 (patients identified through screening programs) (Fasano A, 2001).

In all patients, CD diagnosis was based on serum EMA and anti-tTG positivity associated with evidence of intestinal villi damage (villous height/crypt depth ratio < 3), and in all cases on a subsequent GFD clinical symptoms disappeared and EMA and anti-tTG became negative.

Furthermore, 14 adult patients (8 F, 6 M, median age 36.5 years, range 21-70 years) with symptoms compatible with CD diagnosis but negative for serum EMA and anti-tTG assays were enrolled. They were being investigated for the presence of one or more of the following symptoms: weight loss or failure to thrive, anemia, chronic diarrhea, or abdominal pain. All underwent EGDS with duodenal histology examination, oral mucosa biopsy and EMA and anti-tTG assays in both the duodenal and oral mucosa culture medium. All showed normal histology of the intestinal mucosa and were considered control subjects. In these cases, the complete diagnostic work-up may also have included routine hemato-chemical assays, a thyroid study, serum autoantibodies assay, abdominal ultrasonography and/or computed tomography, colonoscopy, small-intestine barium examination, H2 breath test, duodenal fluid microbiological evaluation and bone marrow biopsy.

All the adult patients included in the study were followed as outpatients, whereas the children were hospitalized.

The protocol was approved by the Ethics Committee of the University Hospital of Palermo and informed consent was obtained from the patients involved in the study or from the parents in the case of the pediatric patients.

Serology for CD diagnosis

Serum IgA was measured by ELISA to exclude IgA deficiency. IgA EMA and anti-tTGs were assayed with commercial kits as previously described[123].

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Intestinal and oral biopsies and histology study

Following general anesthesia (in children) or conscious sedation with meperidine/pethidine 1 mg/kg i.v. and midazolam 0.05-0.1 mg/kg i.v. (in adults), EGDS were performed with standard pediatric or adult video-colonoscopes (Olympus or Fujinon or Pentax, Japan). Six duodenal biopsy samples were obtained from each patient. Four samples underwent routine histology evaluation, and 2 specimens were cultured for 72 h at 37 °C with a commercial reagent set (anti Endomysium-biopsy, Eurospital), as described previously[124,125]. One sample was cultured in the presence of the 31–43 gliadin peptide (0.1 g/L) and the other without its addition. Culture supernatants were collected and stored at -80 °C until used. IgA EmA antibodies in undiluted supernatants were determined by the same commercial reagent set used for serum EMA.

IgA anti-tTG antibodies were determined with a commercial ELISA as recently described[124]. In brief, recombinant h-tTG antigen diluted in PBS was used to coat the wells. The culture medium was diluted 1:5. The conjugate was diluted to obtain reliable OD. Absorbance was read in a microplate reader at 450 nm. Anti-tTG values in the supernatants were expressed as optical density (OD). Normal values were taken as < 300, representing a value > 2 SD above the mean of 200 healthy individuals. The intra-assay CV (coefficient of variation) for the IgA h-tTG autoantibody ELISA on culture medium was 4.2% (n = 20), and the inter-assay CV was 5.9% (*n* = 20).

Oral mucosa biopsy specimens were taken at the same time as the EGDS in children and under local anesthesia (xylocain adrenalin) in adults. The specimens were taken in the mouth, at the second molar tooth region, beneath the occlusal line. Each sample was divided into four parts: two were fixed to be processed for histology evaluation and two were cultured with the procedure described above for the EMA assay in the duodenal mucosa culture medium. Before culture, biopsy samples were weighted (minimal weight required 5 mg/fragment) and washed.

Previous studies in which IgA EMA and anti-tTG antibodies were first assayed on fresh medium and then six months later after storage at -80 °C, had shown that storage did not alter the results obtained, as EMA and anti-tTG results were identical[124,125]. IgA EMA and anti-tTG assays on the culture media were performed by personnel unaware of the clinical and laboratory data of the patients.

Biopsy specimens of the intestinal mucosa adequate in size were immediately oriented with a stereomicroscope and subsequently embedded in paraffin[124,125]. The slides were stained with H&E and graded by conventional histology according to Oberhuber *et al.*[126].

Biopsy specimens of the oral mucosa were embedded in paraffin and the slides stained with H&E; *lamina propria* lymphocytes infiltration was arbitrarily graded as mild when < 10 lymphocytes x 5 HPF (magnification 40X) were counted, and severe when there were > 10 lymphocytes x 5 HPF.

Statistical analysis

We followed the STARD checklist for studies on the diagnostic accuracy of tests[127]. The sensitivity, specificity and diagnostic accuracy values of the diagnostic procedures examined were calculated by standard statistical methods[128]. The Fisher exact test was used to compare the different sensitivity, specificity and diagnostic accuracy values of the assays. The chi-squared test for trend was calculated to compare the frequency of CD cases showing mild, moderate or severe intestinal villous atrophy with or without EMA or anti-tTG positive assays in the culture medium.

Results

None of the patients enrolled showed IgA deficiency. The final diagnoses of the patients with negative serum EmA and normal intestinal histology were: multiple food intolerance (3 cases), Crohn's disease (2 cases), sideropenic anemia (3 cases), major RAS (3 cases), liver cirrhosis (2 cases), duodenal ulcer (1 case).

The results of the IgA EMA and anti-tTG assayed on the duodenal and oral mucosa culture were identical and remained unchanged when the 31-43 peptide was added to the culture medium. A cross-tabulation of the EMA assay results on the culture medium of the oral and duodenal biopsies obtained in pediatric and adult patients, according to the final diagnoses, is shown in Table XXII.

Table XXII. *Cross-tabulation of the EMA assay results on the culture medium of the duodenal and oral biopsies, in adult and pediatric subjects, according to the final diagnoses.*

EmA ASSAY ON DUODENAL MUCOSA CULTURE MEDIUM			
	CD diagnosis (*n*)	Non CD diagnosis (*n*)	Total number
ADULTS			
Positive test	15	0	15
Negative test	0	14	14
Total	15	14	29
CHILDREN			
Positive test	13	0	13
Negative test	0	0	0
Total	13	0	13

EmA ASSAY ON ORAL MUCOSA CULTURE MEDIUM			
	CD diagnosis (*n*)	Non CD diagnosis (*n*)	Total number
ADULTS			
Positive test	9	0	9
Negative test	6	14	20
Total	15	14	29
CHILDREN			
Positive test	6	0	6
Negative test	7	0	7
Total	13	0	13

EMA assayed on the duodenal mucosa culture medium gave results completely concordant with the final diagnoses, as they were positive in all CD patients and negative in all patients not suffering from CD. EMA assayed on the oral mucosa culture medium was positive in 15 of the 28 CD patients (53.6%).

Also anti-tTG antibodies assayed on the duodenal mucosa culture medium were positive in all CD patients and negative in all patients not suffering from CD. Anti-tTG antibodies assayed on the oral mucosa culture medium were positive in 16 of the 28 CD patients (57%) (*Figure 10*).

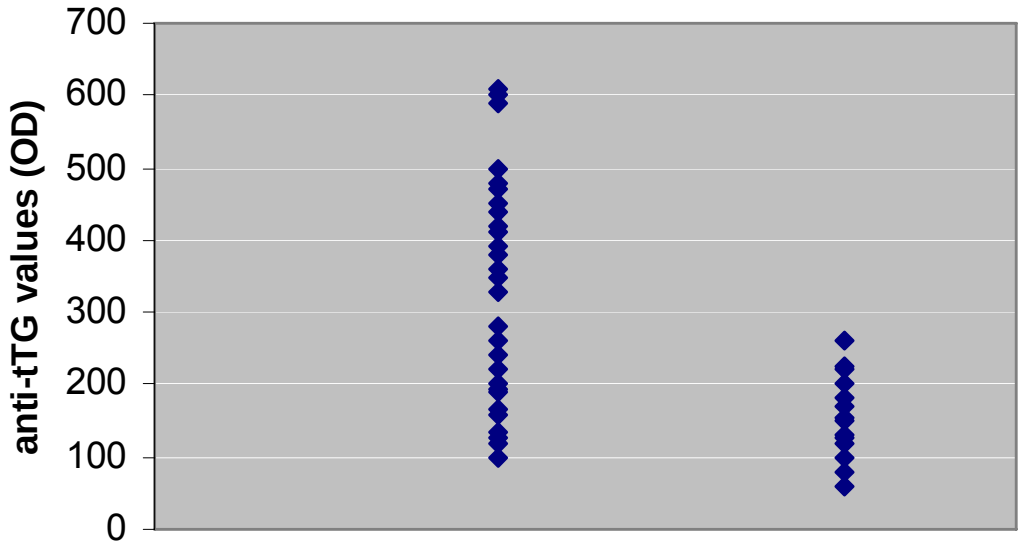

Figure 10. *Individual values of anti-tTG antibodies assayed in the oral mucosa culture medium, in 28 celiac patients and in 14 patients not suffering from CD. Values are expressed as optical density (OD). Dotted line indicates the upper normal limit.*

Table XXIII shows the sensitivity, specificity and diagnostic accuracy of the EmA and anti-tTG assays on the duodenal or oral mucosa culture medium in CD diagnosis.

Table XXIII. *Sensitivity, specificity and diagnostic accuracy in CD diagnosis of the antibodies assays on the culture medium of the duodenal and oral mucosa biopsies. The study groups included 15 adult CD patients, 13 pediatric CD patients and 14 adult controls.*

EMA ASSAY IN DUODENAL MUCOSA CULTURE MEDIUM			
	SENSITIVITY	SPECIFICITY	DIAGNOSTIC ACCURACY
Adults	100%	100%	100%
Children	100%	Not evaluated	Not evaluated
Total	100%	----	----
EMA ASSAY IN ORAL MUCOSA CULTURE MEDIUM			
	SENSITIVITY	SPECIFICITY	DIAGNOSTIC ACCURACY
Adults	60%	100%	79%
Children	46%	Not evaluated	Not evaluated
Total	53.6%	----	----
Anti-tTG ASSAY IN DUODENAL MUCOSA CULTURE MEDIUM			
	SENSITIVITY	SPECIFICITY	DIAGNOSTIC ACCURACY
Adults	100%	100%	100%
Children	100%	Not evaluated	Not evaluated
Total	100%	----	----
Anti-tTG ASSAY IN ORAL MUCOSA CULTURE MEDIUM			
	SENSITIVITY	SPECIFICITY	DIAGNOSTIC ACCURACY
Adults	60 %	100 %	79 %
Children	54 %	Not evaluated	Not evaluated
Total	57 %	----	----

As shown in Table XXIV, CD clinical presentation did not influence the behavior of the EMA and anti-tTG assays in the oral mucosa culture medium. The presence of oral mucosa lesions did not influence the EMA and anti-tTG assays on the oral biopsy culture medium either. In fact, we observed ALU in four CD patients and in four adult controls (three with final diagnosis of major ALU and one with Crohn's disease); only one of the four CD patients and none of these four controls were positive for EMA and anti-tTG antibodies.

TableXXIV. *Results of EmA and anti-tTG assays in the oral mucosa culture medium according to the different clinical presentations in 28 CD patients.*

	Number of EmA positive assays	Number of EmA negative assays
Typical CD presentation	7 cases	7 cases
Atypical CD presentation	7 cases	5 cases
Silent CD	1 case	1 case
	Number of anti-tTG positive assays	Number of anti-tTG negative assays
Typical CD presentation	8 cases	6 cases
Atypical CD presentation	7 cases	5 cases
Silent CD	1 case	1 case

Note: The CD clinical presentation was considered typical when there was malabsorption syndrome, atypical in the absence of intestinal symptoms, and silent in the absence of symptoms.

Figure 11 shows the number of *lamina propria* lymphocytes in the oral mucosa of CD patients and controls. A greater severity of inflammation of the oral mucosa was observed in the CD patients with positive EmA or anti-tTG in the oral mucosa culture medium (Table XXV). In fact, the frequency of positive assays was significantly higher in the patients with severe inflammation: $p<.01$ (Fisher's test).

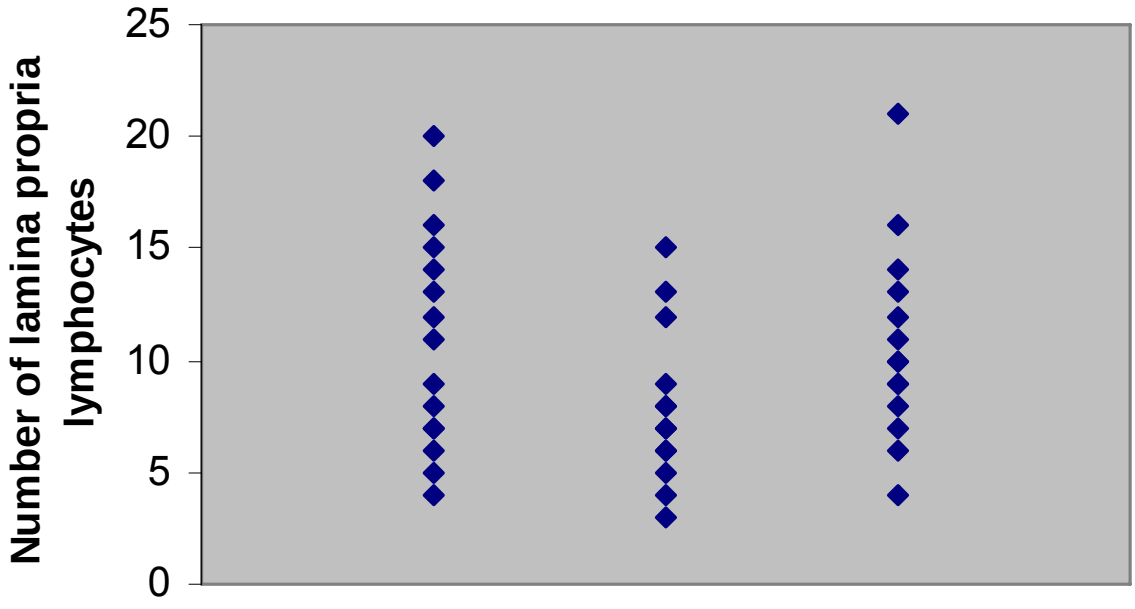

Figure 11. *Number of the lamina propria lymphocytes in the oral mucosa in fifteen CD patients with positive EMA assay in the oral mucosa culture medium (GROUP 1), in thirteen CD patients with negative EMA assay in the oral mucosa culture medium (GROUP 2) and in fourteen patients without celiac disease (GROUP 3). Lines indicate the mean values.*

Table XXV. *Results of EmA and anti-tTG assays in the oral mucosa culture medium according to the severity of the lymphocytes infiltration in the lamina propria of the oral mucosa.*

	Number of EmA positive assays	Number of EmA negative assays
Mild inflammation	7 cases	10 cases
Severe inflammation	8 cases	3 cases
	Number of anti-tTG positive assays	Number of anti-tTG negative assays
Mild inflammation	7 cases	10 cases
Severe inflammation	9 cases	2 cases

Notes: 1) Lamina propria lymphocytes infiltration was graded as mild when ≤ 10 lymphocytes x 5 HPF (magnification 40X) were counted, and severe when there were > 10 lymphocytes x 5 HPF.2) The frequency of EmA positive assays was significantly higher in the patients with severe inflammation: P<.01 (Fisher's test).

Discussion

Although nowadays there is general agreement that CD must be considered a systemic disease and may affect several extra-intestinal organs, the intestinal mucosa examination is still considered the corner stone of a definitive CD diagnosis[2,129]. However, the small bowel biopsy is an invasive procedure which many patients – especially if asymptomatic or suffering from only mild symptoms – find hard to accept. In this respect, a study of the potential diagnostic use of the oral mucosa could be of great interest. In fact, as reported in several sections of this thesis, the oral mucosa is very often damaged in CD[130] and previous studies in patients with CD on GFD showed that both the oral supramucosal application and submucosal injection of gliadin powder or gliadin peptides induced significant immunological changes[106].

As previous studies have demonstrated that EMA and anti-tTG assays on duodenal mucosa culture medium have an excellent diagnostic accuracy in CD diagnosis[124,125,131,132] we aimed to evaluate the ability of the oral mucosa in patients with CD to produce EMA and anti-tTG in a culture system, comparing results with those obtained on duodenal mucosa culture.

Our results showed that the oral mucosa of patients with a new CD diagnosis, still on a gluten-containing diet, was able to produce EMA and anti-tTG in the culture medium, with or without *in vitro* gliadin stimulation. In fact, EMA and anti-tTG assays were positive in 15 and 16 out of 28 patients with CD respectively. This result is in keeping with the theory that the mouth is part of the GALT system[95] and, although its epithelium is very different from that of the gut, the dissemination of the immune effector cells involves the oral mucosa and can determine a gliadin-induced response in patients with CD.

However, in general, the sensitivity of CD-specific antibodies assay in the oral mucosa culture medium was low, with EMA assay more sensitive in adults (60%) than in children (46%). One child negative for EMA assay on oral culture medium resulted to be positive for anti-tTG assay. The sensitivity of the assays was not influenced by clinical presentation as it did not differ between patients with typical, atypical or silent disease. Furthermore, the

presence of “macroscopic” oral manifestations was not relevant as four CD patients were suffering from ALU at the moment of the study but only one of these was positive for both EMA and anti-tTG assays on oral mucosa culture medium. There was a positive correlation between severity of the lymphocites infiltration in the *lamina propria* of the oral mucosa and EmA or anti-tTG positivity in the culture medium. This aspect is similar to the well-known observation that there is a positive correlation between the severity of intestinal histology and the presence of serum EMA in CD[133,134].

EMA and anti-tTG assays on the oral mucosa culture medium showed a specificity of 100%, as we did not observe false positive results. It must be underlined that even the presence of ALU – present in four controls – did not determine false positive EMA/anti-tTG results. Obviously, as we included few control patients, further studies are needed to confirm the absolute specificity of the EmA or anti-tTG assays on the oral mucosa culture medium.

Subsequently to the publication of our results[135], Vetrano *et al.*[136] showed that EMA and anti-tTG autoantibodies were positive in 15/16 and in 15/16, respectively, cultured oral mucosa samples. However, the sensitivity in our oral biopsies was low with 54% for EMA and 57% for anti-tTG, in contrast to 100% sensitivity for anti-tTG described by Vetrano *et al*.[136] These discrepancies could be related to the size of the biopsy samples; in fact, whereas we used biopsy pieces with an approximate weigh of 5mg, Vetrano *et al.*.[136] pointed out that smaller biopsies (< 10 mg) yelded some false negative results, indicating that biopsy size is a limiting factor in the reability of the antibody determination.

As long as this discrepancy cannot completely clarified, caution is advised to suggest the oral mucosa as an area for diagnostic purpose.

However, all the results abovementioned open a new scenario to consider oral mucosa as a new immunological site to detect CD autoantibodies.

A previous study of the oral mucosa histology of untreated CD patients did not show any significant histological alterations which could help diagnosis and the oral gluten challenge performed on CD patients on gluten-free diet showed a sensitivity of 73% and specificity of 80%[137]. However, the immuno-histochemical methods used in previous studies[106,107] were more cumbersome and time-consuming than the simple antibodies assay in the culture medium. Furthermore, the evidence that anti-tTG antibodies can be effectively assayed in the oral mucosa culture medium opens the way to an easy ELISA.

As regards antibodies assay on duodenal mucosa culture medium, our results are in keeping with all the previous reports which have underlined the excellent diagnostic value of this assay[106,107]. The intestine is the main site of EMA production and both EMA or anti-tTG can also be assayed in the whole gut lavage fluid[138] or in the duodenal mucosa by immuno-histochemistry[139,140], giving relevant diagnostic help in cases of seronegative CD patients.

In conclusion, this study demonstrated for the first time that the oral mucosa contributes to EMA and anti-tTG production in untreated CD patients. As the search for easier diagnostic

methods than the small intestine biopsy procedure is important, the antibodies assay on oral mucosa culture medium merits to be further evaluated.

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IMPLICATIONS FOR FURTHER RESEARCHES

The researches conducted in the context of this project, have yielded important results not only in terms of prevalence of oral lesions potentially associated with CD, but above all, thanks to the highly innovative research lines has led to consider the oral cavity, for the possibility to perform easily oral biopsies to be processed with appropriate methods of immunofluorescence and conventional histological examination, as a potential diagnostic gate minimally invasive and easily accessible in comparison with the endoscopic approach. However, clinical trials conducted on largest sample size and on CD patients before and after the GFD, will need to see if the mouth can really be helpful to duodenal endoscopy for the first diagnosis of CD, but also in the follow-up of the disease.

Furthermore, the demonstration that the oral mucosa is a new site involved in the release of pathognomonic autoantibodies of CD and which may show some histological peculiarities offers us a site to obtaine bioptic samples to be underwent cDNA microarrays studies (for genomic studies) and cell and tissue cultures, in order to depth the pathogenesis of CD and to test less toxic compounds, such as different oats species, in the treatment of this condition.

In particular, the potentiality of the oral mucosa to directely react immunologically against the gluten ingested should be taken into account in the development of new therapeutic strategies that aim to prevent the triggering of such immune reactions. Among these new non-dietary therapies under study, the most attractive seems to be the use of enzymes that digest immunogenic gluten peptides; in the event that these peptides could already elicit an immune response in the oral cavity, enzymatic therapies could be ineffective or not fully effective, unless they are already active already in the mouth and not only in the stomach and/or in the small intestine.

Our prelimary results showing that a gluten-related immune response could already be induced in the oral cavity should be taken into account in the development of new non-dietary therapies that aim to prevent the triggering of such immune reactions only at the gastrointestinal level.

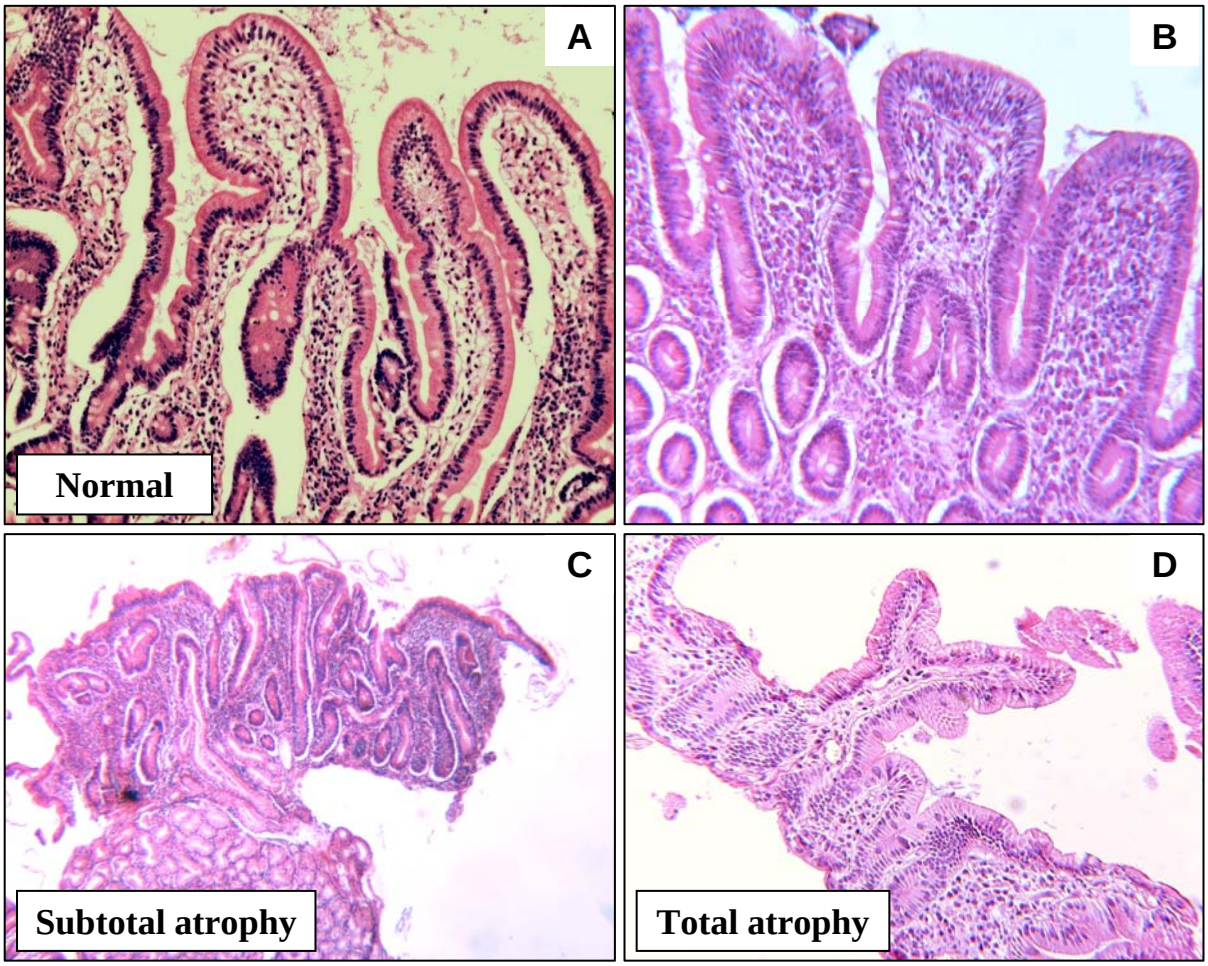

Figure 1. ***Histological features of small intestinal mucosa in patients with untreated CD. (A) Normal mucosa. (B) Partial villous atrophy. (C) subtotal villous atrophy. (D) Total villous atrophy.***

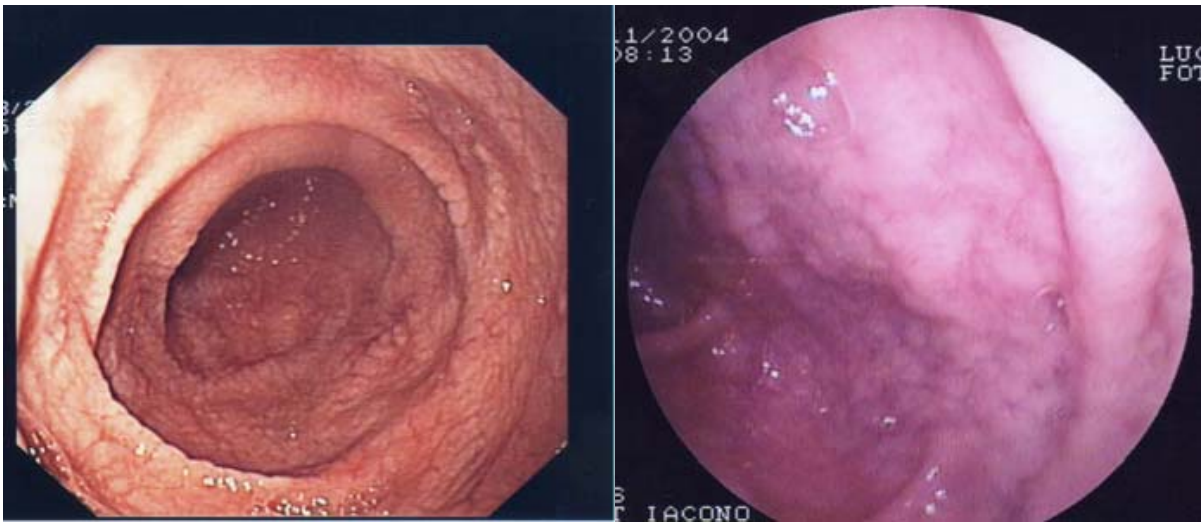

Figure 2. ***Endoscopic signs of villous atrophy. Please note the scalloping and reduction of the duodenal mucosal folds, associated to a mosaic pattern of the mucosa.***

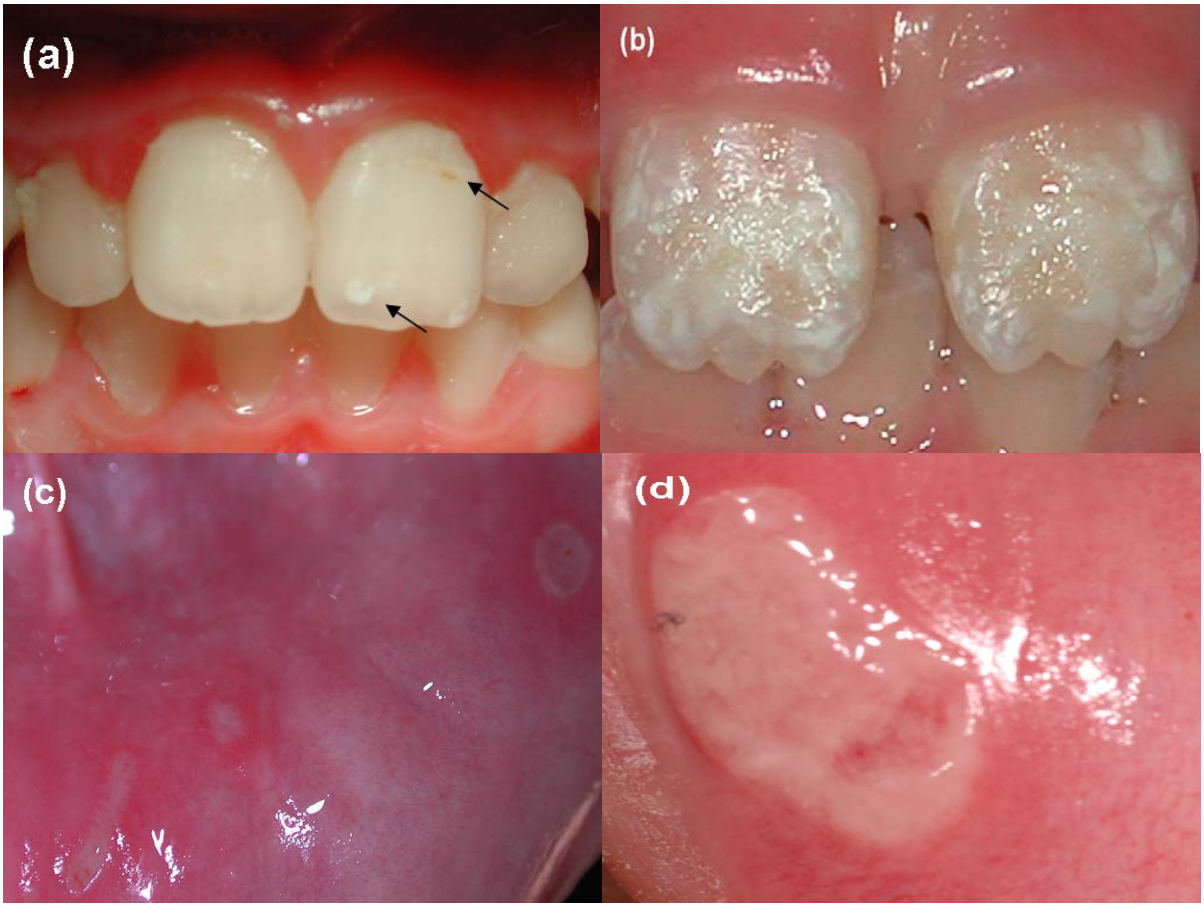

Figure 3. ***(a) Enamel defects of upper incisors (arrows indicate the color changes); (b) Enamel defects of upper incisors (structural changes); (d) major type of ALU and (c) numerous minor-type lesions of ALU.***

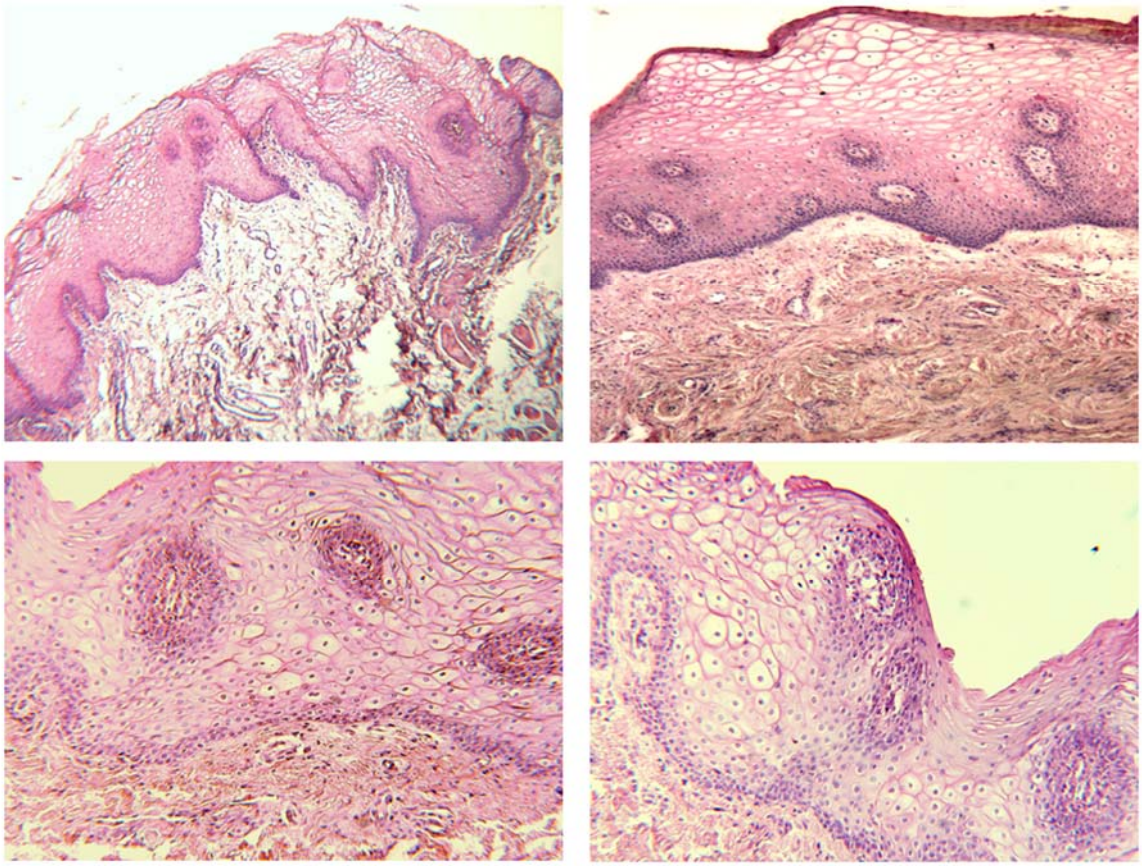

Figure 4. ***Histological features of healthy oral mucosa in different celiac patients. Diffuse distension and vacuolation (spongiosis) of the intermediate and superficial acanthocytes and their overlapping.***

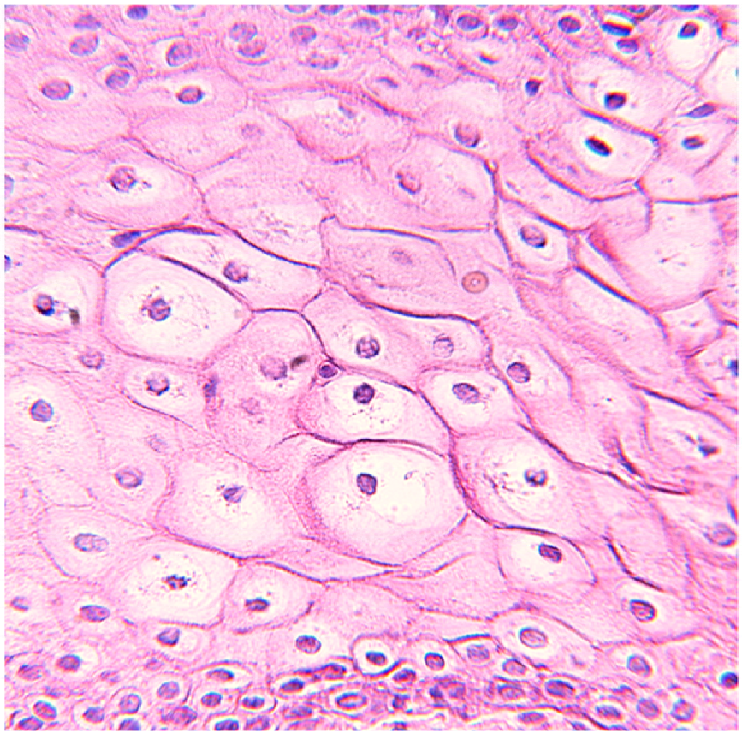

Figure 5. ***Marked overlapping of the acanthocytes***

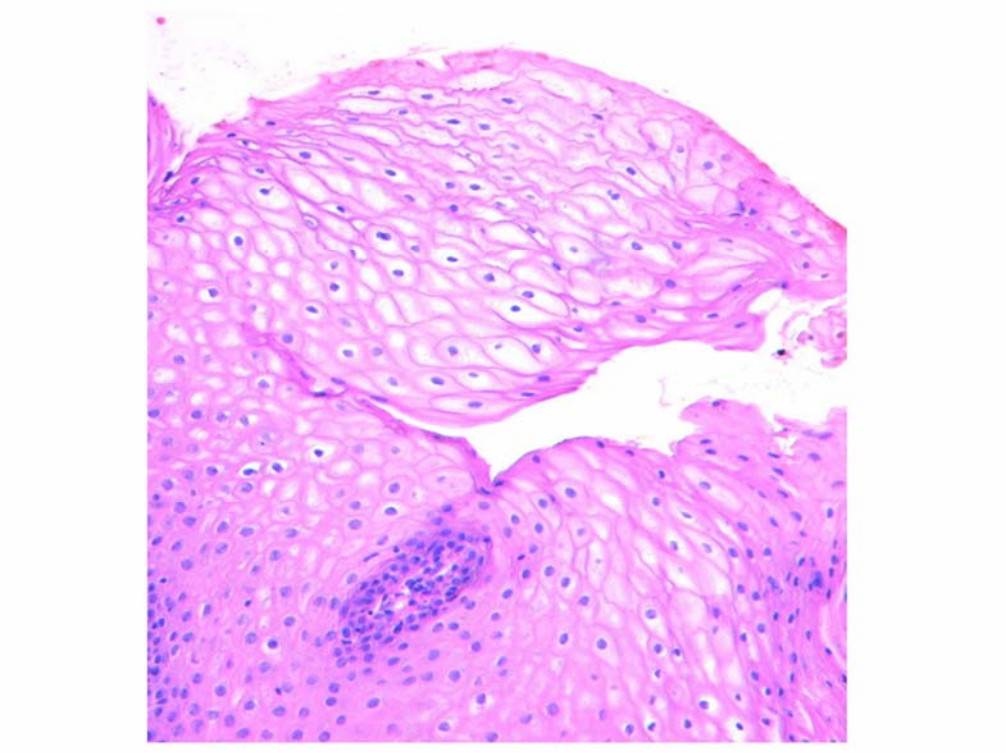

Figure 6. ***Absence of PAS-positive substances (e.g., acid mucopolysaccharides) within the cytoplasm of the keratinocytes with spongiotic aspect.***

ACKNOWLEDGEMENTS

I would like to thank all persons that support my *PhD* studies in particular Prof. A. Craxì and Dr. V. Di Marco (**Gastroenterology and Hepatology Unit, University Hospital of Palermo**) who have had a central role for the recruitment of CD patients, for the study design and for their active cooperation in the realization of scientific products related to this research; Dr. G. Iacono (**First Division of Pediatric Gastroenterology, ''G. Di Cristina'' Hospital of Palermo**) who contributed to the recruitment of paediatric patients; Prof. E. Maresi (**Department of Human Pathology, University Hospital of Palermo**) who performed the histo-pathological analyses.

The Author acknowledges the support of Prof. T. Takata and Prof. Y. Kudo of the **Department of Oral Pathology - University Faculty of Dentistry** (Hiroshima, Japan). Futhermore, a special thank to Prof. M. D'Angelo and to my collegues Dr. C. Paderni, Dr. A. Musciotto, Dr. N. Termine, Dr. O. Di Fede, Dr. V. Panzarella, Dr. C. Di Liberto and Dr. A. Albanese.

Finally, a special thank goes to my *PhD* Tutor, Prof. A. Carroccio and Prof. G. Campisi (supervisor) for their patience and perseverance during the *PhD* course. Their constant support and precious suggestions have been essential to the success of the present project and to my professional growth.