“FEDERICO II”

UNIVERSITY OF NAPLES, ITALY

Faculty of Medicine and Surgery

Ph. D. Program

“Human Reproduction, Development and Growth “

Academic Years 2007-2008

Ph. D. Thesis

The Spectrum of Coeliac Disease: Clinical and Immunological Features of Potential Coeliac Disease

Tutor

Prof. Riccardo Troncone

Student

Dott.ssa Melissa Borrelli

Director

Prof. Claudio Pignata

A mio padre e mia madre, sostegno e guida,
a mio marito ed ai due miei angeli, Gennaro Maria e Ludovica,
con affetto ed amore.

CONTENTS

SCENTIFIC BACKGROUND

COELIAC DISEASE

Coeliac Disease (CD), also called gluten-sensitive enteropathy, is a permanent intestinal intolerance to dietary wheat gliadin and similar prolamins from rye and barley (the toxicity of oats has been reassessed in recent years), that produces mucosal lesions in genetically susceptible individuals.

Epidemiology

Following the original description that gluten is the agent that causes CD (Dicke WK 1953), a better understanding of the clinical aspects of CD, and the availability of serological tests to detect CD-associated antibodies, have contributed to a significantly improved diagnosis and management of the disease. CD represents one of the most frequent genetic diseases of humankind. Recently, it has become clear that CD is much more prevalent than previously thought, when the reported prevalence of symptomatic CD is l in 1000 live births, with a range from l in 250 (observed in Sweden) to l in 4000 (observed in Denmark) (Greco L 1992). Population based screening studies have clearly shown that CD is underdiagnosed, clinical CD representing the top of the iceberg. In fact, these studies have

demonstrated the worldwide prevalence to be 1 in 266 (Fasano A 2001). In particular, the prevalence of CD is estimated in the range of 1:100 to 1:300 individuals in the European general population. Similar prevalence have been indicated in North Africa, in South America and in USA (Fasano A 2001, Maki M 2003, Ciclitera PJ 2005). In recent study conducted in the United States, Fasano A and colleagues (Fasano 2003) observed that in at risk groups CD occurs in 1 of 22 of first-degree relatives, in 1 of 39 of second-degree relatives and in 1 of 56 in symptomatic patients. The overall prevalence of CD in not-at-risk group is 1:133 (Fasano A 2003). Interestingly, during the last few years many studies report a "new epidemiology of CD", showing that it is common not only in Europe and in people of European ancestry, but also in the developing countries, such as Southern Asia, The Middle East, North West and East Africa, South America, both in the general population and in the groups at risk (Cataldo F 2007).

Genetics

Coeliac disease is a typical multifactorial disease in which both several genetic and environmental factors are needed for the disease onset. The familial clustering is demonstrated by disease prevalence of about 10% among the first degree relatives which is ten fold higher than the risk of CD at the general population level. The prevalence was further corroborated by

two large population-based twin studies in the Italian population, which found a concordance rate in dizygotic twins of around 20% and in monozygotic twins of around 85% (Greco L 2002, Nistico L 2006). The increase in concordance between twins and the fact that monozygotic twins do not have a 100% concordance rate suggest the involvement of environmental factors in addition to genetic factors.

The involvement of HLA complex located in the major histocompatibility complex (MCH) region on chromosome 6, which predisposes to CD, has been known for over 30 years (Falchuk ZM 1972, Stokes PL 1972). In particular, convergent evidence from different populations has suggested that the primary association of CD is with the HLA-DQ2 (coded by alleles DQA1*05, DQB1*02, inherited either in cis or trans) or the HLA-DQ8 heterodimers (coded by alleles DQA1*03, DQB1*0302) with respective DR-DQ haplotypes (Sollid ML 1989, Mantovani V 1993). Among 1008 European CD patients, 88% carried DQ2, 6% were DQ2-DQ8+, 5.6% carried “half” DQ2, i.e. either the DQA1*05 or DQB1*02, and only 0.4% of patients were totally negative for DQ2 and DQ8 coding alleles (Karell K 2003). A gene dosage effect has been suggested, and a molecular hypothesis for such phenomenon has been proposed based on the impact of the number and quality of the HLA DQ2 molecules on gluten peptide presentation to T cells (Karell K 2003, Louka AS 2002). The most likely mechanism to explain the association with HLA

class II genes is, in fact, that the DQ molecule binds a peptide fragment of an antigen involved in the pathogenesis of CD to present it to T cells. DQ2 and DQ8 are, however, common also in the healthy population, each with carrier frequencies of approximately 20-30% among Caucasians. The estimated contribution of the HLA region on developing CD is around 40% (Bevan S 1999), meaning the remaining 60% of genetic factors involved in CD is not well known. Chromosome 5q31-33 region markers (CELIAC2 locus) have shown genetic linkage to coeliac disease in several genome wide analyses and their replication studies. Significant linkage to this chromosomal region was identified in Italian (Greco L 2001), Swedish-Norwegian (Naluai At 2001), Finnish (Liu J 2002), Irish (Zhong F 1996), as well as in Dutch population (van Belzen MJ 2003). This region contains a cytokine gene cluster and it might play a role in immune regulation and inflammation. The CELIAC3 locus on chromosome 2q33 has shown linkage to CD and was replicated by a few but not all the studies performed (Victorien M 2008). The CELIAC 3 locus contains the T lymphocyte regulatory genes CD28, CTLA4 and ICOS. Recently, a novel candidate gene myosin IXb (MYO9B) on chromosome 19p13 (CELIAC4 locus) was found in the Dutch population (Monsuur AJ 2005). Interestingly, MYO9B is a good candidate gene for CD because of its function; it encodes an unconventional myosin molecule that may have a role in actin remodelling of epithelial enterocytes. The gene may therefore be potentially relevant in

intestinal barrier integrity. However, in CD population in the United Kingdom, Spain, Italy and Scandinavia could not be replicated (Victorien M 2008). A new genetic association between factor V Leiden and CD was discovered (Mari T 2006).

With recent advances in genotyping technology, the whole genome association studies with hundreds of thousands SNPs have become feasible. Although they require big sample cohort and are therefore still very expansive to perform, the strategy has revealed already promising results in finding also genes of relatively low risk effects in many complex disease. Recently, the first genome-wide associated study (van Hell DA 2007) and its follow up (Hunt KA 2008) have identified eight new loci that contribute significantly towards coeliac disease risk. Seven of these contain genes controlling adaptive immune response, including IL2/IL21 (4q27), RGS1 (1q31), IL18RAP (2q11-2q12), CCR3 (3p21), IL12A (3q25-3q26), TAGAP (6q25) and SH2B3 (12q24). Wijmenga C and colleagues (Romanos J 2008) in Italian cohort showed that common variation in IL2/IL21, RGS1, IL12A/SCHIP and SH2B3 was associated with susceptibility to coeliac disease. The LPP and TAGAP regions also showed moderate association, whereas there was no association with CCR3 and IL18RAP. These results may imply there is a genuine population difference across Europe regarding the loci contributing to coeliac disease.

However, linkage peaks observed in non-HLA regions are much lower and not consistent compared to HLA. This might be because many non-HLA genes contribute to the pathogenesis of CD. Hence, the contribution of a single predisposing non-HLA gene might be modest.

Clinical presentation

Typical clinical manifestations of CD include chronic diarrhoea, abdominal distension, weight loss, anorexia and irritability. Intestinal symptoms are common in children diagnosed within the first two years of life. Within weeks to months of starting to introduction of gluten in the diet these symptoms can be observed (Fasano A 2005). A coeliac crisis, characterized by explosive watery diarrhea, marked abdominal distension, dehydration and shock, described more commonly at the beginning of this century, is now observed rarely.

CD presenting features later in childhood and in adult patients are characterized by the prevalence of unusual intestinal complaints (e.g. recurrent abdominal pain, nausea, bloating and constipation) and/or extra-digestive symptoms including short stature, delayed puberty, anemia, dental enamel defects, skin lesions, isolated hypertransaminasemia, bone pains and fractures, arthritis, infertility, recurrent fetal loss, and aphthous ulceration. In addition, a variety of neuropsychiatric conditions such as depression, anxiety, peripheral neuropathy, ataxia, epilepsy with or without

cerebral calcifications, and migraine headaches have been reported in individuals with CD. A recent study reports considerable changes in the age at presentation and the presenting features of CD in children over the past 20 years (Ravikumara M 2006). The incidence of CD and the age at diagnosis have risen. Gastrointestinal manifestations as presenting features have decreased dramatically (between 1999 and 2004, only 42% of children had gastrointestinal manifestations, compared with 75%-88% of children diagnosed between 1983-1998), while the symptoms at diagnosis have become fewer and less severe (Ravikumara M 2006). Data from studies on adults are similar, with Lo et al (Lo W 2003) reporting that of 227 patients only 43% presented with diarrhoea after 1993 compared with 73% before 1993.

Different may be the mechanisms operating in these different situations. Such extradigestive manifestations may more likely result from the intestinal damage and consequent nutritional deficiencies (e.g. anemia, osteopenia) and/or due to the deranged (auto?)immune response (e.g. skin, liver, joints, CNS involvement).

Finally, it has become evident, mostly from family studies, that CD may be completely silent clinically (Auricchio S 1988). Large numbers of silent cases of CD have been reported in at-risk groups (such as patients with insulin-dependent diabetes and first-degree relatives) and in general population samples enrolled in screening programs. In paediatric age,

almost 25% of children with CD are diagnosed by targeted screening of high-risk group and this group is symptom free at diagnosis (Ravikumara M 2006). Villous atrophy occasionally may be detected by endoscopy and biopsy conducted for another reason: a recent study reports a prevalence of 1.5% of CD in patients with dyspepsia, a symptom not typically associated with CD, concluding that it may be appropriate to recommend the CD screening for the patients with refractory dyspepsia (Ozaslan E 2007).

Particularly close associations have been reported between coeliac disease and various diseases, many with an autoimmune pathogenesis, such as thyroid diseases (Mulder CJJ 1988), Addison's disease (Reunala T 1987), pernicious anemia (Stene-Larsen G 1988), autoimmune thrombocytopenia (Stenhammar L 1988), sarcoidosis (Douglas ID 1984), insulin-dependent diabetes mellitus (Savilahti E 1986), alopecia (Corazza GR 1995) and cardiomyopathies (Not T 2003). Coeliac disease has been shown to co-exist with Down's (Carlsson A 1998, Bonamico M 2001) and Turner's syndrome (Bonamico M 2002). Further, it has been reported that 1.7-2.6% of coeliac disease patients have selective serum IgA deficiency (Cataldo F 1998). In all these groups silent CD is often present.

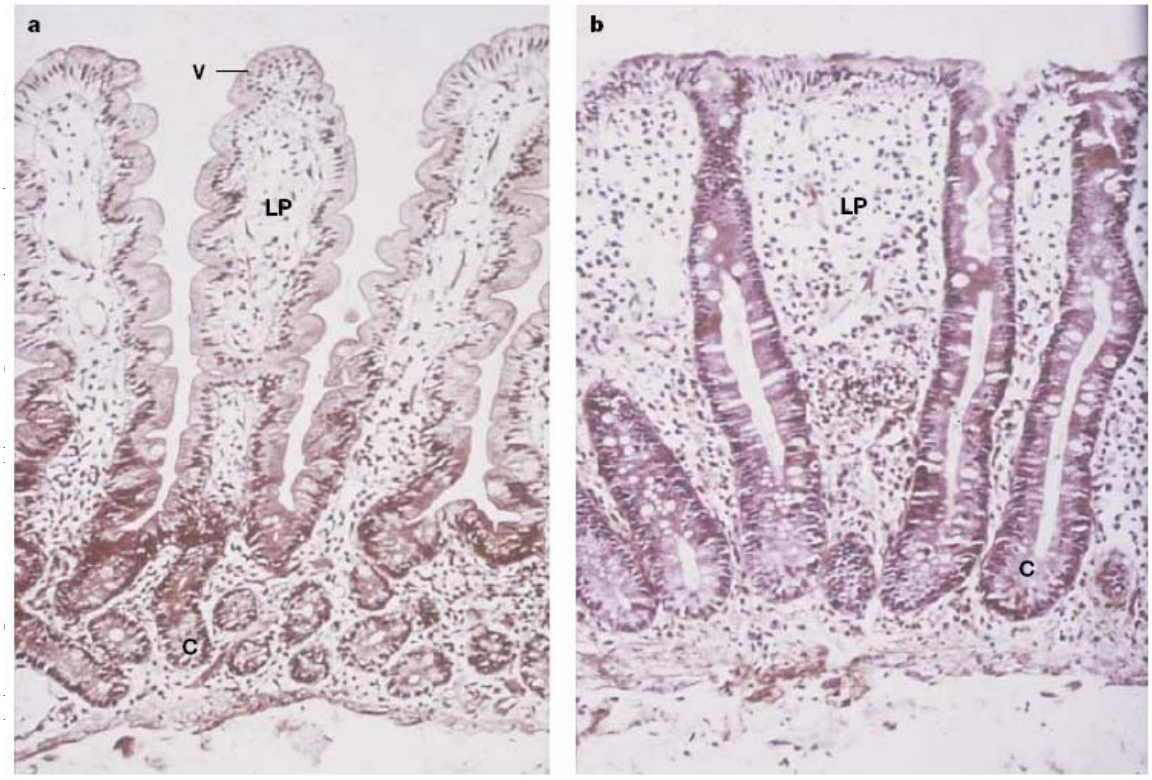
Whether the mode of presentation of CD correlates with the degree of villous atrophy is still controversial. Previous studies have demonstrated an association of severity of histological findings and mode of presentation, in particular the authors concluded that more severe clinical symptoms are

better predictors of a more severe form of small bowel histopathology in children and in young patients (Weizman Z 1997, Tursi A 2002). A recent study, that included many more patients than the previous studies (a cohort consisted of 449 adult CD patients vs 144 and 59 respectively), does not confirm this correlation. The authors, correlating of mode of presentation (classical, diarrhoea predominant or atypical/silent) with histology of duodenal biopsies, concluded that the clinical presentation does not correlate with histological findings (Brar P 2007).

Diagnostic criteria

In 1969, ESPGAN recommended three intestinal biopsies for the diagnosis of CD in childhood: one performed at the time of presentation, another after the patient has been on a gluten-free diet when the mucosa is expected to have returned to normal, and the final biopsy after the patient has been rechallenged with gluten, when villous atrophy is expected to have recurred (Meeuwisse G 1970). Twenty years later a working group from the ESPGAN reconsidered such diagnostic criteria (Working Group of ESPGAN 1990). The two requirements mandatory for the diagnosis of CD remain: 1) the finding of villous atrophy with hyperplasia of the crypts and abnormal surface epithelium (Fig. 1), while the patient is eating adequate amounts of gluten; and 2) a full clinical remission after withdrawal of gluten from the diet. The finding of circulating IgA

antibodies to gliadin, reticulin, and endomysium at the time of diagnosis, and their disappearance on a gluten-free diet, adds weight to the diagnosis. A control biopsy to verify the consequences on the mucosal architecture of the gluten-free diet is considered mandatory only in patients with equivocal clinical response to the diet and in patients asymptomatic at first presentation (as is often the case in patients diagnosed during screening programs, e.g., first-degree relatives of coeliac patients) (Working Group of ESPGAN 1990).



a

FIG.1 (A) Hematoxylin-eosin (H&E)-stained section of a normal small intestinal biopsy. It shows villi (V) and crypts (C). the lamina propria (LP) extends between the crypts and into the core of each villous, and it contains a rich vascular and lymphatic network into which digestive products are absorbed. Original magnification, x 400. **(B)** H&E-stained section of a small intestinal mucosa biopsy from an

individual with CD. Note villous atrophy, enlarged hyperplastic crypts and increased infiltration of lymphoid cells in lamina propria and epithelium.

Challenge is not considered mandatory, except under unusual circumstances. These include situations where there is doubt about the initial diagnosis, for example when no initial biopsy was done, or when the biopsy specimen was inadequate or not typical of CD. Again, the diagnostic gluten provocation may be necessary to exclude other causes of enteropathy, such as cow's milk sensitive enteropathy, postenteritis syndrome, and giardiasis that could be responsible for the flat mucosa. These disorders frequently occur in the first 2 years of life, for this reason gluten challenge is recommended in children younger than 2 years of age gluten provocation (Working Group of ESPGAN 1990). Gluten challenge should be discouraged before the age of 7 years and during the pubertal growth spurt. Once decided, gluten challenge should always been performed under strict medical supervision. It should be preceded by an assessment of mucosal histology and performed with a standard dose of at least 10 g of gluten per day without disrupting established dietary habits. A further biopsy is taken when there is a noticeable clinical relapse or, in any event, after 3 to 6 months. Serologic tests (IgA gliadin, reticulin and endomysium antibodies, absorptive and permeability tests), more than clinical symptoms, can be of help in assessing the timing of the biopsy to shorten the duration of the challenge (Mayer M 1989).

It is now quite widely accepted that the development of the CD mucosal lesion is a dynamic process, so there is a range of histological changes from normal villous architecture with an epithelial lymphocytosis, through partial villous atrophy to total villous atrophy (Marsh MN 1992). In a situation where jejunal histology has lost specificity, and with the growing contribution by serology, and to a less extent by HLA, many propose to move to a new diagnostic approach mainly based on antibodies and genetics.

According to recently published guidelines, serologic testing is of primary importance to screen patients for biopsy and follow response to treatment (Hill ID 2005). IgA anti-human recombinant tissue transglutaminase antibodies (TG2-HR) detected by enzyme-linked immunosorbent assay (ELISA) and IgA endomysial antibodies (EMA) seen on monkey esophagus (ME) or human umbilical cord (HUC) substrates by indirect immunofluorescence are considered the most sensitive and specific serologic test for CD (Hill ID 2005, Rostom A 2005). It is well-established that increased EMA and TG2 in both children and adults correlate with abnormal small bowel histopathology (Rostami K 1999, Sategna-Guidetti C 1993, Abrams JA 2004). Tursi et al (Tursi a 2003) observed in adult people that the prevalence of TG2 and their mean serum value were higher in CD patients with severe enteropathy (Marsh 3b-c) than in those showing slight enteropathy (Marsh 1-3a). In a study of 30 children it is reported a

correlation between TG2 antibody titer and Marsh score (Hoffenberg EJ 2000). Only recently, Donaldson MR et al report levels of serum antibodies that were nearly always associated with severe enteropathy. They confirm that IgA TG2 and EMA levels correlate with duodenal villous atrophy in pediatric CD patients and observe that strongly positive antibodies levels (TG2 >100 units and/or EMA titer >1:1280) are highly specific (>98%) for Marsh 3a or greater lesions (Donaldson MR 2007).

Because more than 98% of people with coeliac disease share the major histocompatibility complex II class HLA-DQ2 or DQ8 haplotype, the inclusion of HLA typing for these haplotypes is useful, especially in particular situations. In fact, HLA-DQ2 OR HLA-DQ8, or both, are found in approximately 40 percent of the general population, but in more than 99 percent of coeliac patients. People who do not have HLA-DQ2 or HLA-DQ8 haplotypes are unlikely to have coeliac disease (Kaukinen K et al 2002).

In conclusion, considering that more than 40% of the population has the HLA alleles implicated in coeliac disease and more than 1% a positive serology, with an increasing number of subjects showing signs of minor enteropathy, the analysis of jejunal biopsies is still very important instrument for the diagnosis of coeliac disease. So, until serological methods are improved, the genetic make up of coeliac patients is better defined, it seems wise for a diagnosis of coeliac disease still rely on a

combined approach based of clinical criteria, histology, serology and genetics.

"Potential" Coeliac Disease

The development of the CD mucosal lesion is a dynamic process that may present in various stages or types of lesions and in duodenal biopsy with a normal architecture, an increase in the number of IEL can be the first and most sensitive index of the effects of gluten on the mucosa and can be therefore the single most important histological feature in CD (Marsh MN 1992). It is well known that intraepithelial lymphocytosis are increased in the mucosa of untreated coeliac patients, but these cells are not pathognomic for CD, as their density can be elevated on other disorders, such as autoimmune diseases and in patients that use nonsteroidal anti-inflammatory drugs. However, about in nearly 10% of cases can be the initial presentation of CD (Kakar S 2003). Certainly, an increase of $\delta\gamma$ + IELs in the presence of villous atrophy or villous tip IELs (severe partial villous atrophy) strengthens the probability of CD in borderline cases where the histology is difficult to interpret (Jarvinen TT 2003, Jarvinen TT 2004).

Ferguson introduced the terms of silent, latent and potential CD (Ferguson A 1993) indicating subjects with no symptoms, but severe mucosal damage (silent), and subjects with positive antibodies but a normal, or almost normal, jejunal mucosa (latent and potential), with latent being

different from potential patients, as the former had already shown, at least once in their life severe gluten-dependent villous atrophy. Thus, the potential CD is a patient has anti-endomysium antibodies (EMA) and/or anti-human tissue transglutaminase antibodies (TG2), the typical HLA-predisposing genotype (DQ2 or DQ8), but a normal or minimally abnormal mucosa architecture (increased intraepithelial count) at the intestinal biopsy examination. Potential CD patients present a high count of intraepithelial lymphocytes and subtle pathological alteration such as increased density of intraepithelial lymphocytes expressing γδ TCR, signs of activated mucosal cell-mediated immunity (such as expression of CD25 and B7 by lamina propria mononuclear cells), coeliac-like intestinal antibody pattern, and positive rectal gluten challenge (Troncone R 1996, Paparo F 2005). Several studies investigated antibody pattern in small intestinal aspirate to characterize CD-associated markers, that could identify latent/potential CD patients. The coeliac-like antibody pattern in jejunal fluid was observed in patients with clinical symptoms of CD but with normal jejunal biopsy histology and some of these patients were clinically gluten sensitive (Arranz E 1993). Similarly patients with irritable bowel syndrome (IBS) had increased CD associated antibodies in duodenal aspirate and were HLA-DQ2 positive. Interestingly, stool frequency and duodenal antibodies decreased significantly under a gluten-free diet in the subgroups of HLA-DQ2-positive and intestinal antibody-positive IBS patients (Wahnschaffe U

2001). In the recent study with the aim to identify a marker to detect coeliac disease before the development of villous atrophy, the authors demonstrate that intestinal transglutaminase autoantibodies deposits have a sensitivity and specificity of 93% in detecting subsequent coeliac disease (Salmi TT 2006).

GUT IMMUNE SYSTEM

In the gut is a delicate balance between the need to recognise pathogens and to prevent unwanted immune responses to food antigens or the normal intestinal flora, yet allowing adequate nutrient uptake at the same time. Considering the large area of the gastrointestinal tract, it is not surprising that it has developed both immunologic and non-immunologic ways of protection. The mucosa barrier consists of intestinal epithelial cells connected by tight junctions and non-immunologic defence mechanisms such as low pH, peristalsis, and mucus coat, all together protecting the intestine from invading antigens (Sanderson IR 1999).

The intestinal lymphoid tissue is the largest compartment of the immune system in the body and referred to as the gut-associated lymphoid tissue (GALT). It consists of mesenteric lymph nodes, Peyer's patches, isolated lymph follicles and large numbers of lymphocytes scattered throughout the lamina propria and epithelium of the intestine (MacDonald TT 2003).

Human regulatory T cell analysis in the gut

The immune system controls activation of the innate as well as the adaptive arms through various means, primarily including induction of anergy apoptosis of activated immune cell and the activities of regulatory CD4+ T cells. In addition, several other regulatory mechanism are

operational in the gut mucosa, including CD8+ T cells, γδ+ T cells and NKT cells, that are highly correlated with their surrounding epithelial cells, and IL 10 secreting B cells, immature dendritic cells and plasmacytoid dendritic cells.

It is becoming increasingly clear that the most important among these regulatory cells reside within the CD4+ T cell population. These cells play an important role in induce and maintaining peripheral self-tolerance and thus preventing immune pathologies. Several types of regulatory T cell (Tregs) have been identified and the mechanisms of suppression may different. They can be divided into two major groups, the so-called "naturally occurring" regulatory T cells and "adaptive" regulatory T cells, that include Th3, Tr1 and CD8+ T cells (Fig. 2; Tab. 1).

"Naturally occurring" regulatory T cells. Most CD4+ T cells that recognise autoantigens in the thymus with high affinity are either clonally deleted or differentiate into a "naturally occurring" Treg. The thymus-derived naturally occuring CD4+ T cells comprise 1-2% of human peripheral CD4+ T cells and 5-10% of mouse (Fig. 2; Tab. 1). These cells are selected in the thymus and thus have a pre-defined antigen specificity (Sakaguchi S 2004, Shevach EM 2004). More recently, many groups have found that CD4+CD25+ Treg cells can also be induced in the periphery from naïve precursors (Walker MR 2003). Thymus derived Treg, initially, were identified by their CD4+CD25^{high} phenotype but an increasing

number of markers has been recently reported. These cells also express intra-cytoplasmic and cell-surface CTLA-4 (CD152), which is a negative regulator of cell-mediated immunity, and GITR (Glucocorticoid-induced Tumor necrosis factor(TNF) receptor family-Related), which is a member of the TNF receptor superfamily. CD25, as well as CTLA-4 and GITR can also be observed on activated non-regulatory T cells, so these molecules does not functionally define the Treg cell. A significant advance in more precisely defining the Treg population occurred when the forkhesd trascription factor FOXP3 was identified as being necessary for Treg development. Mutations in the Foxp3 (murine)/ FOXP3 (human) gene were identified as the sole genetic defect underlying the phenotypes of the fatal autoimmune diseases of the scurfy mouse (Bruskow ME 2001) and human IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) a rare monogenic disease of male children that is accompanied by autoimmune disease, such as type 1 diabetes, IBD, and severe allergy (Bennett CL 2001). The foxp3 gene was identified as a master regulatory gene; it is constitutively and specifically expressed in natural Treg and plays an indispensable role in their development and function. The specificity of foxp3 in mice is clear; it is solely expressed in Treg and the scurfy mutation is always related to defective suppressive function. Conversely, the expression of FOXP3 in humans is not restricted to Treg and can be induced on activation of conventional T cel, albeit at

much lower levels than in natural Treg. Recent research has demonstrated that CD127 expression is down-modulated on Treg cells (Liu W 2006). CD127 is part of the heterodimeric IL-7 receptor that is composed of the CD127 and the common γ chain, which is shared by other cytokine receptors (IL2R, IL4R, IL9R, IL15R and IL21R). CD 127 is expressed on thymocytes, T and B cell progenitors, mature T cell, monocytes, and some other lymphoid and myeloid cells. Studies have shown that the CD127 plays an important role in the proliferation and differentiation of mature T cells, and *in vitro* experiments show that the expression of CD127 is down-regulated following T cell activation. The correlation of down-regulated CD127 on T cells with high-to-intermediate expression of CD25 on Treg cells was confirmed by intracellular staining of Foxp3 antigen to identify Treg cell (Liu W 2006, Seddiki N 2006).

Human Treg can be greatly expanded ex vivo by TCR stimulation in the presence of high concentrations of IL-2 and CD25 is functionally essential as a key component of the high affinity IL2 receptor. The exact mechanism of suppression by Treg remains uncertain. In vitro studies has shown that the proliferation of the responder T cells and their cytokine production is strongly suppressed in the presence of Treg. The suppressive function is cell contact dependent and independent of cytokines (Earle KE 2005, Setoguchi R 2006). However, mouse studies have proven the suppression to be dependent on cytokines such as transforming growth factor (TGF)β

and IL10. The mechanisms responsible for these differences between *in vivo* and *in vitro* results remain to be fully explained.

"Adaptive" regulatory T cells. Apart from the CD4+ CD25+ thymus derived Treg, other types of Treg cells that can develop in the periphery are the Tr1 and Th3 types of regulatory cells (Fig. 2; Tab. 1). T regulatory type 1 cells are induced by an IL10–dependent process *in vitro* and *in vivo.* They are defined by their capacity to produce high levels of IL 10 and TGF β and IL5, low amounts of IFN γ and IL2 and no IL4. Several investigators have demonstrated that Tr1 cells do not constitutively express FOXP3 unlike of naturally occurring CD4+CD25+ Treg cells, but upon activation it can be up-regulated to levels similar to those observed in activated CD4+CD25- T cells (Vieira PL 2004, Levings MK 2005) Tr1 cells regulate immune responses through the secretion the immunosuppressive cytokines IL 10 and TGF β, and they suppress both naïve and memory T cell responses *in vivo* and *in vitro*. Although Ag-specific Tr1 cells need to be activated via the TCR in order to exert their suppressive function, once activated, Tr1 cells can mediate bystander suppressive activity against other Ags. This bystander suppression is likely mediated by the local release of IL10 and TGF β which act on both APC and T cells. IL10 down regulates expression of co-stimulatory molecules and pro-inflammatory cytokine production by APC and directly inhibits IL2 and TNF α production by CD4+ T cells (Groux H 2003, Pestka S 2004). Similarly, TGF β down-regulates the

function of APC and inhibits proliferation and cytokine production by T cells (Strobl H 1999, Cerwenka A 1999). The suppressive effects of Tr1 cells are typically either partially or fully reversed by addition of anti-IL10 and anti-TGF β neutralizing mAb, but additional mechanisms may also contribute.

Th3 progenitor cells are similar to the Tr1 cells in that they are also CD4+CD25-Foxp3- (Fig. 2; Tab. 1). Th3 cells secrete mainly TGF-β and to a lesser extent IL4 and IL10 (Chen 1994, Fukaura 1996).

IL10-secreting Tr1 or TGFβ-secreting Th3 cells, may also express Foxp3 (Levings MK 2002), suggesting that the T regulatory cells do not constitute separate lineages.

CD8+ Tr1 like cells, with produce significant amounts of IL10, low IFNγ and no IL4, IL5 and nor TGFβ, have also been described (Fig. 2) (Gilliet M 2002, Steinbrink K 1999).CD8+ T regulatory cells are less well characterized and are reportedly capable of suppressing CD4+ cells *in vitro.* This conclusion is based upon inhibition of proliferation of CD4 T cell during the stimulation of CD8+ T regulatory cells with anti CD3 antibodies. In this case, the regulation involves a cell-cell contact-dependant mechanism. These cells also display the commonly accepted markers for Treg cells; CD25 and Foxp3, the latter of which is induced upon anti-CD3 stimulation (Bisikirska B 2005).

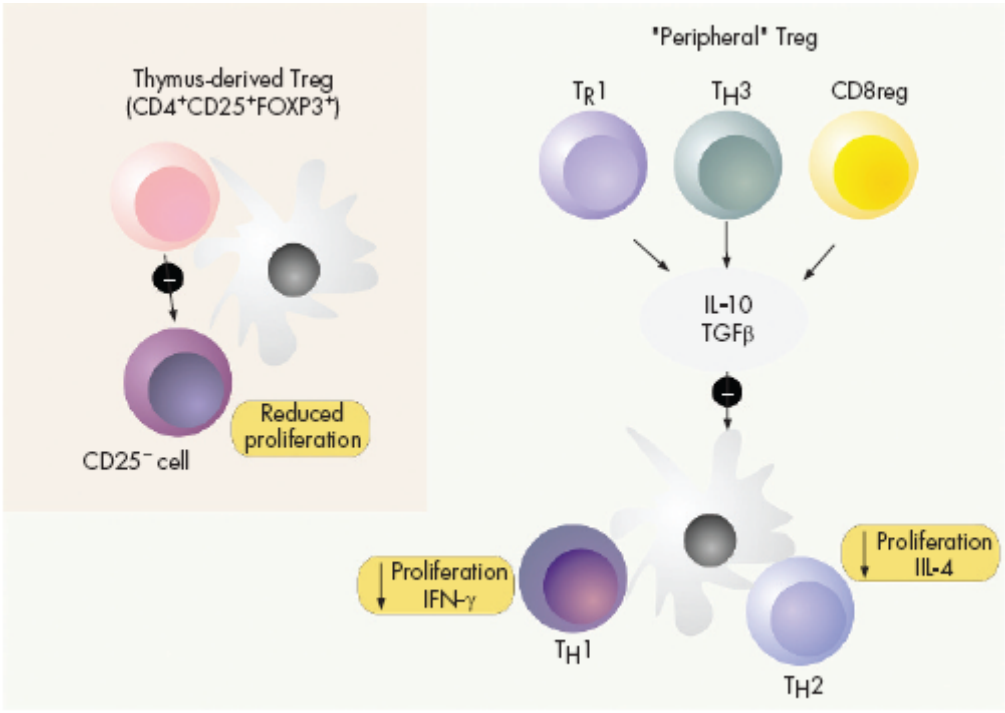


Fig. 2 Regulatory T cells and their function. Several types of regulatory T cells have been identified and the mechanisms of suppression may differ. Thymus derived regulatory T cells, also known as naturally occurring regulatory T cells, are a subset of CD4+CD25+ T cells and are thought to suppress activation of T cells at level of antigen presenting cell. Adaptive peripheral induced regulatory T cells include Tr1, Th3 and CD8. These cells produce the immunosuppressive cytokines IL10 and TGFβ and function in a cytokine dependent manner (Huibregtse IL 2007).

Table 1 Characteristics of the different regulatory T cell subsets

Feature	Naturally Treg	Adaptive Treg	
Subpopulations	$CD4^+CD25^+$	Tr1	Th3
Site of induction	Thymus	Periphery	
Mechanism of action	Cell–cell contact, cytokine independent	Cytokine dependent	
Characterisation	$CD25^+$ and $Foxp3^+$	IL-10	TGF-β
Specificity	Self-antigens in the thymus	Tissue specific antigens and foreign antigens	
Protection demonstrated	Transfer colitis, SCID	Transfer colitis, SCID	Neutralising TGF-β antibodies

IL, interleukin; TGF-β, transforming growth factor β; Treg, regulatory T cells.

Cytokines

An immune response to an invading pathogen is an essential mechanism to provide specific defence against any particular antigen. Regulation of the magnitude and duration of this response is essential to prevent hypersensitivity and/or autoimmune reactions. This immunoregulation is controlled by soluble factors which perform the functions of the immune response and mediate signalling between the various cells involved. Such families of regulatory molecules include the cytokines and their antagonists, immunoglobulins, serum proteins, complement and other inflammatory mediators.

Cytokines are a family of low molecular weight protein regulatory molecules involved in cell growth, inflammation, immunity, differentiation and repair. They are characterized by their pleiotropic activity, acting on a wide range of cell types, and, unlike hormones which are carried by the bloodstream throughout the body, cytokines generally act in a local manner through autocrine and paracrine mechanisms. Cytokines mediate their effects through binding to their receptors, which activates intracellular signals (Ho IC 2002). Any cytokine may have many different biological effects depending on the target cell, and different cytokines may have similar effects. Cytokines are not generally produced constitutively by cells, and once activated are tightly regulated. It is now accepted that T

helper (Th) lymphocytes, which are required for both cell-mediated and humoral immune response, are composed of two distinct subsets, Th1 and Th2, distinguished by different patterns of cytokines production (Mosmann TR et al 1986), and the type of response they elicit in target cells expressing cytokine-specific receptors (Kim J 1985). Th1 cells support CD8+ cytotoxic cells by production of IL2, macrophage activation and MCH class II expression by production of Interferon (IFN)-γ. They also support delayed type hypersensitivity (DTH) response, and immunoglobulin switching to IgG2a, and produce tumor necrosis factor (TNF)-β. The Th2 cells secretory pattern is characterized by IL4, IL5, IL6, IL10 and IL13 cytokines; they also contribute to B-cell activation, switching to the IG1 (in rat but not in humans), IgA and IgE isotypes and to antibody production. Th0 subset, postulated to be either precursors of Th1 and Th2 cells or itself a final differential phenotype, is characterized by production of cytokines of both Th1 and Th2 types (Mosmann TR et al 1986, De Carli M 1994). The dose of the antigen, strength of the signal through the TCR, and costimulation all influence on the Th differentiation, but the most potent determinant is the cytokine milieu itself around the cell, in particular IL 12 drives differentiation toward a Th1 and IL4 toward Th2 (Ho IC 2002). Although a distinct Th1/Th2 cytokine profile is not as clear in humans as in animal cells, an inverse relation remains between the tendency of T cells to produce IFNγ as opposed to IL4 and IL5

(MacDonald TT 2001).

Although CD4 T helper cells are the major sources of IL2, IFNγ and IL4, IL13, respectively, other cell types are able to produce these cytokines. Subsets of γ/δ cells and CD8+ cells can secrete Th1 or Th2 like cytokines pattern. NK cells produce IFNγ and TGFα and contribute to the Th1 like response. IL4 is syntetized by macrophages, keratinocytes and B cells (Mosmann TR 1996). Cytokines are usually divided into proinflammatory(IL1, IL2, IL6, IL12, IL18, IFNγ and TNFα), anti-inflammatory (IL4 and IL13) and immunosuppressive (IL10 and TGFβ), based on their activity.

PATHOGENESIS OF THE COELIAC DISEASE

CD results from the interaction between gluten and immune, genetic, and environmental factors. CD is induced by the ingestion of gluten, which activates immune responses in the small intestine that lead to the destruction of the epithelial mucosa and subsequently cause deleterious effects on intestinal absorption of nutrients. For a long time it was thought that only the adaptive immunity plays a role in CD. It is now clear that both the adaptive and the innate immune responses are important in CD and that some gluten peptides seem to be involved in either one of these responses. This is also reflected by several gluten peptides having distinct pathological mechanisms in CD (Jabri B 2005).

The role of gluten

Gluten is derived from wheat, barley and rye. From a nutritional standpoint, it is a rather poor nutrient that provides elasticity and viscosity to food (Wieser H 1996). The term "gluten" refers to entire protein component of wheat; it is a mixture of gliadin and glutenin proteins. The gliadin is the alcohol-soluble fraction of gluten, it is a complex mixture of 40 highly homologue proteins that are divided according to their electrophoresis mobility into 3 families: α/β-, γ- and ω-gliadins (Wieser H 1996); the glutenins consist of low molecular weight (LMW)- and high molecular weight (HMW)-glutenins. A large numbers of studies have

shown that both gliadin and glutenins contain numerous immunogenic peptides (Kagnoff MF 2007).

Gluten and related proteins are also known as prolamins for the high content of amino acids such as glutamine (Q)(40%) and proline (P)(20%). The high proline content renders these proteins resistant to complete proteolytic digestion by gastric, pancreatic, and brush border enzymes in the human intestine, since those enzymes are deficient in prolyl endopeptidase activity (Shan L 2005). Thus, relatively large peptide fragments with a high proline and glutamine content remain in the intestinal lumen after gluten ingestion (Shan L 2002). Nonetheless, the relatively poor digestion of these proteins alone is not sufficient to cause CD; there is no known difference between healthy individuals and those susceptible to developing CD in their ability to digest these proteins. The decrease in gluten digestion by brush border enzymes (propyl endopeptidase (PREP) and pyroglutamyl-peptidase I (PGPEPI)), observed in untreated CD patients when compared to controls seems to be secondary to impaired structure of the intestine and not causal. In fact, the two genes that encode for peptidase enzymes are also located in CD linkage regions (6q21-22 and MYO9B) and have been studied for their potential causal role in CD, but no association could be found (Monsuur AJ 2006).

Experimental evidence has also indicated a direct, non immune-mediated, cellular toxicity of gliadin-derived peptides *in vitro* on both cell

culture systems and mucosal explants. For example, a peptic-tryptic digest of gliadin (PT-gliadin) can agglutinate *in vitro* the K562 (subclone S) cell line and can interfere with the differentiation of fetal rat intestine, in addition to being capable of direct damage for the intestinal mucosa (Gianfrani C 2008). Furthermore, in cultures of coeliac small intestinal mucosa tissue (an *in vitro* model for the study of CD) the presence of PT-gliadin or the short peptide 31-43 can specifically prevent a restitution of enterocyte height that would normally occur within 24-48 hours (De Ritis G 1988). Finally, PT-gliadin and the 31-43 peptide exert a direct cytotoxic effect on epithelial Caco2 cells through the induction of *fas*-mediated apoptosis and a delayed inactivation of the EGF receptor, phenomena that favour cell growth in the crypts of the atrophic mucosa of CD patients (Barone M 2007, Giovannini C 2000).

Permeability of the epithelial barrier in CD

The activation of immune response in the gut implies that gliadin and/or its breakdown peptides in someway cross the intestinal epithelial barrier and reach the lamina propria of the intestinal mucosa where they are recognised by antigen presenting cells (APCs). Despite the progress made in understanding the immunological aspects of the pathogenesis of CD, the early steps that allow gliadin to cross the intestinal barrier are still largely unknown. Under physiological circumstances the intestinal epithelial

barrier is described as being almost impermeable to macromolecules. During such healthy states, quantitatively small but immunologically significant fractions of antigens cross the defence barrier. These antigens are absorbed across the mucosa along two functional pathways. The vast majority of absorbed proteins (up to 90%) cross the intestinal barrier through the transcellular pathway, followed by lysosomal degradation that converts proteins into smaller non-immunogenic peptides. The remaining portion of proteins is transported as intact molecules, resulting in antigen specific immune responses. This latter phenomenon utilises the paracellular pathway that involves subtle but sophisticated regulation of intercellular tight junction (tj) that can contribute to antigen oral tolerance (Fasano A 1998). When the integrity of the tj system is compromised (such as during prematurity or exposure to radiation, chemotherapy and toxins) an immune response to environmental antigens, including autoimmune diseases and food allergies, may develop (Fasano A 2001). A important modulator of tj permeability is zonulin. Zonulin has a molecular weight of 47 kDa, an N terminal receptor binding motif that is structurally and functionally similar to the zonula occludens toxin (Zot) elaborated by *Vibrio cholerae*, and a C terminal domain probably involved in the rearrangement of cytoskeletal elements functionally connected to intercellular tj (Fasano A 2001). Following binding to its surface receptor, zonulin induces a protein kinase C (PKC) mediated polymerisation of intracellular actin filaments which are

directly connected to structural proteins of the tj (Fig. 3). The complex actin cytoskeleton network of the enterocyte is known to be involved in the intracellular trafficking of molecules as well as in the regulation of paracellular permeability by its direct interaction with the tj structural protein (Wang W 2000).

In CD the intestinal permeability is increased in untreated CD patients but still present in treated patients and healthy relatives (Schulzke JD 1995, Uil JJ 1996). Increased permeability can be observed even in patients with dermatitis herpetiformis (DH) and no evidence of histological damage in biopsy specimens (Smecuol E 2005).

In particular, in acute CD the crypt tj showed aberrant morphology, and in asymptomatic patients treated with the gluten-free diet, jejunal tj structure only partially recovered (Schulzke JD 1998). This could be mediated by zonulin, in fact recently it is demonstrated that zonulin expression is chronically up-regulated in CD gut tissues (Fasano A 2000, Smecuol E 2005).

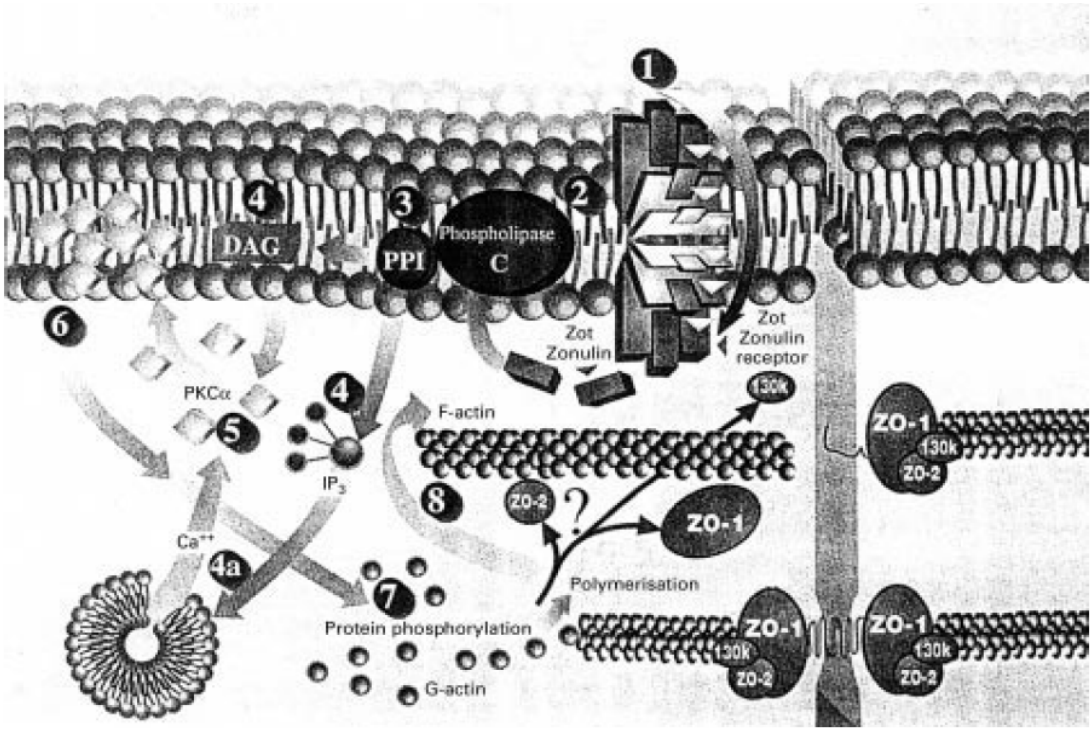


Fig. 3 Proposed zonulin/zonula occludens toxin (Zot) intracellular signalling leading to the opening of intestinal tight junctions. Zonulin and Zot interact with the same specific surface receptor (1) whose distribution within the intestine varies. The proteins are then internalised and activate phospholipase C (2) that hydrolyses phosphatidyl inositol (3) to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG) (4). Protein kinase C (PKC) is then activated (5), either directly (via DAG) (4) or through release of intracellular Ca^{++} (via PPI-3) (4a). PKC catalyses the phosphorylation of target proteins with subsequent polymerisation of soluble G-actin to F-actin (7). This polymerisation causes the rearrangement of the filaments of actin and the subsequent displacement of protein (including ZO-1) from the junctional complex (Fasano A 2001).

The adaptive immune response

The adaptive immune response involves gliadin-reactive CD4+ T cells in the lamina propria that recognize gliadin peptides, which are bound to HLA class II molecules DQ2 or DQ8 on antigen-presenting cells (APCs). Gluten-specific CD4+ T lymphocytes can be isolated from small intestinal biopsies of CD patients but not from controls (Sollid LM 2005).

How HLA-DQ2 and HLA-DQ8 bind such peptides was an enigma for several years, because the peptide-binding groove of HLA-DQ2 and DQ8 favors the binding of peptides with negatively charged residues at key anchor positions. Such negatively charged amino acids are largely absent from native "gluten" peptides generated in the human intestinal tract. However, this puzzle was solved after the discovery that the target antigen of an autoantibody present in many CD patients was a calcium-dependent tissue transglutaminase (TG2). Tissue TG2, witch is released in the intestinal mucosa during tissue injury, has a role in tissue repair and cross-links proteins by forming isopeptide bonds between glutamine and lysine residues. In fact, the most important function of TG2 is to catalyze post-traslational modification of proteins by transamidation or deamidation. In particular, deamidation occurs in the absence of suitable amines involves water and occurs at lower pH. It results in the transformation of neutral glutamine into negatively charged glutamic acid. Gliadin is an excellent

TG2 substrate because of its high glutamine and proline content (Molberg O 1998). Thus, deamidation plays an important role in favoring the binding of gliadin peptides to DQ2 or DQ8 molecules, which possess binding pockets that have a preference for negatively charged amino acid residues. Only particular glutamine residues, however, are modified by TG2. Vader LW and colleagues (Vader LW 2002) have shown that the spacing between glutamines and proline determines which glutamines in gluten will be modified in the process of deamidation. Several distinct gluten T cell epitopes exist and they have been found in α-gliadins, γ-gliadins and, recently, in ω-gliadins (Gianfrani C 2008). A single 33-mer peptide has the highest T-cell stimulatory capacity in a large cohort of CD patients. This peptide maps at the level of the 56-88 residues of α-gliadin and harbors six copies of three different T-cell epitopes (Shan L 2002). This 33-mer fragment is rich in proline; proline affects gluten immunogenicity by inferring resistance to gut proteolysis, by its importance for TG2 specificity and by dictating binding of the peptides to the HLA molecules. Nonetheless, HLA-DQ2-restricted T cells from children with CD recognize also "gluten" peptide that do not contain deamidated glutamine residues; this indicates that glutamine deamidation is not required for the initiation of an anti-gliadin immune response (Vader W 2002). Perhaps, deamidation plays a role, not in allowing an anti-gluten response to take place but altering the nature of the anti-gliadin response. So, deamidation may

reshape the anti-gliadin response by favoring the binding to DQ molecules and by amplifying the immune response (Jabri B 2005) (Fig. 4).

In addition to deamidation, TG2 can also cross-link glutamine residues of peptides to lysine residues in other proteins, including itself. It is possible that complexes between gluten and TG2 permit gluten reactive T cells to provide help to TG2 specific B cells enabling them switch their antibody production from IgM to IgA or IgG by a mechanism of intramolecular help (Sollid LM 1997). There are several studies which have addressed whether the anti-TG2 antibodies are involved in development of the coeliac lesion in the gut. Deposition of TG2 antibodies to extracellular fibrinogen bound TG2 is an early sign in development of CD, and this can be seen with the sign of overt pathology in the gut (Salmi TT 2006). The antibodies have weak inhibitory effect on the transamidating activity of TG2 *in vitro*, but this inhibitory effect is probably insufficient to affect the in vivo enzymatic function of TG2 (Esposito C 2002; Dieterich W 2003). Serum IgA of CD patients have been found to inhibit epithelial cell differentiation in a co-culture model of fibroblasts and epithelial cells (Halttunen T 1999). These are interesting lead observations, but further knowledge is necessary to conclude whether the anti TG2 antibodies are involved in the immunopathogenesis of CD.

Upon their activation, the gluten reactive T cells produce cytokines of which IFNγ is clearly dominating. Paradoxically, the major Th1 inducing

cytokine, IL12, has not been detected in the CD mucosa (Nilsen 1995, Nilesen 1998). IL18 (Salvati VM 2002) and IFNα (Monteleone G 2001) are both candidate as driving factors for Th1 differentiation and IFNγ production. An increased mRNA expression of IL6, IL1β, TNFα, IL2 and TNFβ was found in the small intestinal mucosa of untreated CD patients, whereas no differences in IL4 production was observed. There were results discordant for expression of IL10 and TGFβ (Lahat N 1999, Kontakou M 1995, Beckett CG 1996, Lionetti 1999, Salvati VM 1999).

The signalling pathway related to gamma interferon has also been explored: persistent STAT1 activation was observed (Mazzarella G 2003) as well as enhanced interferon regulations factor 1 (IRF1) expression (Salvati VM 2003). IL10 is also increased, but not enough to control the strong gamma IFN production (Forsberg G 2002). Downstream T cell activation, a complex remodelling of the mucosa takes place, involving increased levels of metalloproteinases (Daum S 1999) and growth factors (Salvati VM 2001) which leads to the classical flat mucosa.

The innate immune response

It is clear that the activation of CD4+ T cells is not sufficient to explain the destruction of surface epithelial cells, the dramatic increase in IELs, and their malignant transformation. All these characteristic features of CD are not found in other intestinal diseases, such as Crohn's disease and

autoimmune enteropathies, that are associated with CD4+ T cell activation and high levels of IFNγ expression in the lamina propria. Recent studies suggest that activation of the innate immune system is important in the pathogenesis of CD and in some of the complications of this disease, namely in refractory CD and in development of enteropathy-associated T cell lymphomas (EATLs). In particular, an increase in the number of IELs in the mucosa of the small intestine is a characteristic feature of CD, and these cells are likely to be important for the ongoing pathogenesis of CD (Jabri B 2000). Following activation, IELs from patients with CD change from being typical antigen-specific T cells to being NK-like cells able to mediate epithelial damage through the recognition of stress-induced molecules on intestinal epithelial cells (Meresse B 2006). IELs, in fact, expressing the T cell receptor (TCR) and CD8 can also express NK cell receptors (NKRs) like NKG2D, CD94/NKG2A and CD94/NKG2C. The cytokine IL15 takes center stage in this process. Gluten induces epithelial cell stress that results in the up-regulation of IL15 and non-classical MHC class I molecules. In particular, IL15 induces increased expression on intestinal epithelial cells of epithelial cell surface ligands, such as MIC (ligand for NKG2D), while HLA-E (ligand for CD94/NKG2A and CD94/NKG2C) can be induced by IFNγ. These molecules up-regulate the activating NKRs of epithelial effector cytotoxic T cells leading to reduce the TCR activation threshold and mediate direct killing of epithelial cells

expressing the appropriate non-classical MCH class I ligands (MIC and HLA-E) (Jabri B 2007). Thus, the reduction of the TCR activation threshold and the arming of NKRs to mediate direct killing in IELs can explain how IELs can kill epithelial cells without recognizing gliadin peptides. Activation of intraepithelial lymphocytes is likely to contribute to epithelial cell apoptosis and villous atrophy via FAS/FAS ligand (Maiuri L 2001) and perforin-granzyme (Ciccocioppo R 2000) pathways. Because arming of NKRs by IL15 to kill in an antigen non-specific manner can only occur in effector T cells, the questions of what is the initial TCR trigger of IEL activation and whether this trigger is specific to coeliac disease are raised (Fig. 4).

Several studies have reported that gluten can induce a stress response in epithelial cells and these effects have also been linked with effects of gluten on innate immune cells like macrophages and dendritic cells (DCs) (Maiuri L 2003). Peptides in the amino-terminus of α-gliadin (p31-49 and p31-43) are a prototype gluten fragment which may exert such effects. Independently by binding to HLA-DQ2 or HLA-DQ8, they are able to up-regulate IL15 production by intestinal epithelial cells, to increase IEL infiltration and epithelial cells apoptosis in human intestinal mucosa organ culture model, and to cause epithelial damage when instilled into the human duodenum (Hue S 2004, Maiuri L 2003). This findings raise another question: why does “gluten” not induce similar responses and epithelial cell

damage in everyone's small intestine? Notwithstanding their auto-reactivity, the intraepithelial T cells with NKRs seem to be under the control of gluten reactive CD4 positive cells. This argument is based on the observation that HLA is a necessary genetic factor for the development of CD. Still the mechanism for this crosstalk between the gluten reactive CD4+ T in the lamina propria and intraepithelial CD8+ T cells expressing NKRs is currently unknown. However, neo-expression of the NKR ligand HLA-E by epithelial cells as a function of CD4 T cell produced IFNγ offers an attractive possibility.

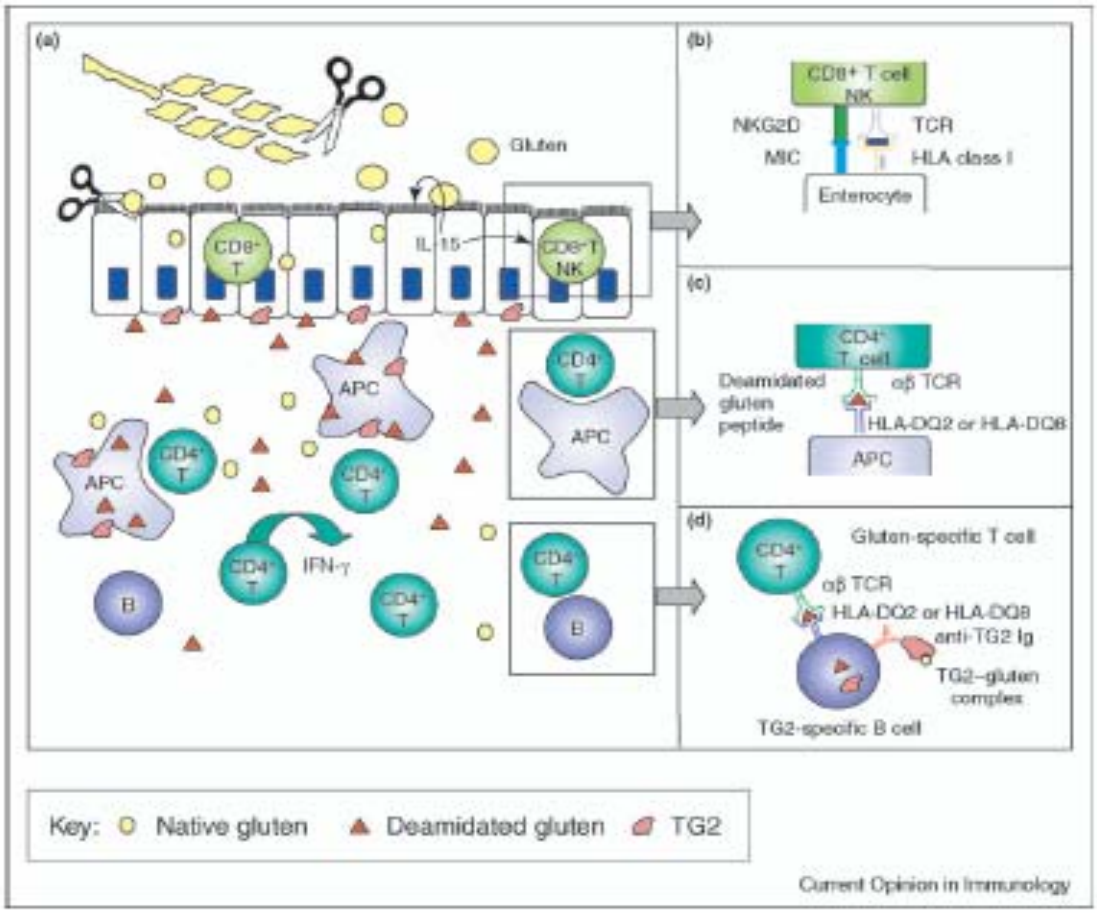


Fig. 4 (a) the parts of the gluten proteins that are resistant to processing by luminal and brush border enzymes survive digestion and are transported across the epithelial barrier as polypeptides. Gluten peptides are deamidated by TG2. CD4+ T cells in the lamina propria

recognize predominantly deamidated gluten peptides, presented by HLA-DQ2 or DQ8 molecules on the cell surface of APC. In the epithelium there is infiltration of CD8+ T cells that express NK cell receptors (NKRs), such as NKG2D. in the lamina propria there are B cells specific for gluten and TG2. **(b)** Intraepithelial T cells, by up-regulation of NKG2D, can kill enterocytes expressing MIC molecules either by reducing the TCR activation threshold or by mediating direct killing. Gluten can induce NKG2D and MIC expression by stimulating the expression of IL15. **(c)** Hla-DQ2 and DQ8 molecules have a preference for binding peptides with negatively charged amino acids and thereby bind gluten peptides deamidated by TG2 with increased affinities. **(d)** a model of how gluten-reactive T cells control the formation of antibodies to TG2 by intramolecular help. This can happen in the lamina propria or, more likely, in the mesenteric lymph nodes. The complexes are endocytosed and degraded in endosomes where gluten peptides are released for binding to DQ2 or DQ8 molecules. After transport of DQ2/DQ(with bound peptides to the cell surface, gluten-reactive T cells can recognize the gluten peptides and threby provide T cell help to the TG2-specific B cell. (Sollid LM 2005).

RESEARCH PROJECT

This thesis reports the results I obtained during the Doctorate in "Human Reproduction, Development and Growth " (XXI Cycle) from 2005-2008. During the past 3 years, my research has been focused in the clinical and molecular characterization of CD and in particular of potential CD.

In particular the following lines were developed:

1. The coeliac disease diagnostic criteria.
2. Spectrum of coeliac disease: "potential" coeliac disease.
3. Immunologic markers in potential coeliac disease: cytokines and T regulatory cells.
4. Immunologic markers in coeliac patients with selective IgA deficiency.

1. THE COELIAC DISEASE DIAGNOSTIC CRITERIA

The first objective of my research project was to investigate the diagnostic methods used by Italian pediatricians in their diagnostic approaches to CD.

In 1990, current criteria followed for the diagnosis of CD were introduced by the members of European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (Working Group of ESPGAN 1990). These criteria are based on two main requirements: the finding of villous atrophy with hyperplasia of the crypts and abnormal surface epithelium, while the patient is eating adequate amounts of gluten; and a full clinical remission after withdrawal of gluten from the diet. The finding of circulating IgA antibodies to gliadin, reticulin, and endomysium at the time of diagnosis, and their disappearance on a gluten-free diet, adds weight to the diagnosis (Working Group of ESPGAN 1990).

Since the introduction of the revised ESPGAN criteria in 1990, serological methods have greatly improved with the introduction of anti-tissue TG2 antibodies measurement (Troncone R 1999). Similarly HLA typing has been increasingly used in clinical practice (Sacchetti L 2001). At the same time, the awareness of different clinical manifestations of coeliac disease has evolved, as well as the recognition of a wide spectrum of histological alterations.

In a situation where jejunal histology has lost specificity, and with the growing contribution by serology, and to a less extent by HLA, I believe, in according with many, that the current diagnostic criteria for CD need a revision. Starting from this supposition, this study was designed in order to create the basis for a possible revision of the 1990 ESPGHAN guidelines.

In this study a nationwide questionnaire on current diagnostic routines was sent by mail to 54 Italian centres for CD diagnosis. The questionnaire contained 12 multiple choice questions, divided in three sections: the first section regarded the serological tests used in the centres, the HLA typing and immunohistochemistry, if performed; the second section was focused on the diagnostic criteria currently used in symptomatic and asymptomatic children; the third section concerned the clinical management in some special cases, such as children below the age of 2 years, children with positive serology and normal mucosal architecture (potential CD), and IgA deficient patients.

The results of this study, reported in detail in the following enclosed paper, provided that in all the centres anti-TG2 measurement is routinely performed, followed by EMA to confirm the results. In a smaller number of cases, an AGA antibody measurements is still used. 87.5% of the centers perform HLA typing. In most cases the test is used to ascertain susceptibility to develop the disease, especially in high risk patients, such as first degree relatives, but it is also used in unclear cases mainly to

exclude the possibility of CD. Up to 67.5% of centres consider an infiltrative lesion (Marsh 1) consistent with CD diagnosis; interestingly, 15% will make a diagnosis of CD even with completely normal mucosa, if patients are symptomatic and have a positive serological analysis. In dubious cases, 80% of centers perform immunohistochemical analysis; but only 5% analyze γδ+ intraepithelial lymphocytes. The gluten challenge is performed in children younger than 2 years in only 20% of the Centers and more than 80% do not perform a second biopsy to verify the effects of a gluten-free diet in clinically silent patients, as recommended by the 1990 criteria.

Despite the new diagnostic tools available, ESPGHAN criteria are still widely followed by Italian diagnostic Centers; the only exception regards the gluten challenge, which is not performed in children younger than 2 years and in clinically silent patients, as recommended by the 1990 criteria.

The question if the intestinal biopsy is or not necessary remain still open. It is well-established that increased EMA and TG2 in both children and adults correlate with abnormal small bowel histopathology (Tursi A 2003, Donaldson MR 2007). Thus, the patients with high titers of anti-TG2 antibodies, HLA DQ2 or DQ8 positivity and gluten-dependent symptoms almost invariably have a villous atrophy (Green PH 2003); in these cases diagnosis could probably be established without small bowel biopsy (Kaukinen K 2001). But, it is now clear that CD encompasses a large

spectrum of clinical manifestations that range from serious symptomatic forms to completely asymptomatic forms. At the same time, from a histological point of view, it goes from the typical severe lesions of duodenal mucosa to forms characterized by minor degrees of enteropathy (Marsh MN 1992).

The patients with minor degrees of enteropathy (potential or early-developing CD) do not fulfill the traditional ESPGHAN diagnostic criteria. In these case, the contribution of the analysis of jejunal biopsies may still be very important to define the disease state. In particular, when CD is suspected, but histology is not diagnostic increased density of γδ lymphocytes (Spencer J 1991) and villous tip IEL (Jarvinen TT 2004) supports the diagnosis of CD. Few centers, however, are able to perform such an analysis since it requires frozen biopsies.

In conclusion, until serological methods are improved, the genetic make up of coeliac patients is better defined, it seems wise for a diagnosis of coeliac disease still rely on a combined approach based of clinical criteria, histology, serology and genetics. Moreover, a revision of the current ESPGHAN criteria might be useful, as has already been frequently suggested, but it should be evidence-based. Large, multicenter studies are greatly needed.

PAPER SUBMITTED AND ACCEPTED BY JPGN:

THE COELIAC DISEASE DIAGNOSTIC METHODS OF THE ITALIAN PEDIATRICIANS.

Renata Auricchio, Viviana Granata, Melissa Borrelli, Riccardo Troncone and on behalf of the Italian Society for Pediatric Gastroenterology Hepatology and Nutrition (SIGENP).

Department of Pediatrics & European Laboratory for the Investigation of Food-Induced Diseases, University Federico II, Naples

Editorial Manager(tm) for Journal of Pediatric Gastroenterology & Nutrition
Manuscript Draft

Manuscript Number: JPGN-NA-08-151R1

Title: THE COELIAC DISEASE DIAGNOSTIC APPROACH OF THE ITALIAN PEDIATRICIANS

Article Type: Short Communication

Section/Category: Gastroenterology

Keywords: Celiac disease; diagnosis

Corresponding Author: Dr. Renata Auricchio, PhD

Corresponding Author's Institution: Department of Pediatrics

First Author: Renata Auricchio, PhD,MD

Order of Authors: Renata Auricchio, PhD,MD; Viviana Granata, MD; Melissa Borelli, MD; Riccardo Troncone, PhD, MD

Manuscript Region of Origin: ITALY

Abstract: The aim of this study was to investigate the current implementation of the 1990 ESPGHAN criteria for the diagnosis celiac disease (CD) in Italy , in order to form a foundation for their revision.
From September 2006 to March 2007 a nationwide questionnaire concerning current diagnostic methods was sent by mail to 54 Italian centers for the diagnosis of CD, which were distributed across the entire national territory. The questionnaire investigated the tests performed, diagnostic criteria currently used and the management of some special cases in each center.
80% of the centers use anti-tissue transglutaminase to diagnose CD, and anti-endomysium antibodies, to confirm the results. 55% still use anti-gliadin antibodies. 87.5% of centers perform HLA typing, especially in first degree relatives and in unclear diagnosis. Regarding histology, 67.5%

of centers consider an infiltrative lesion consistent with diagnosis of CD. The majority of centers (85%) use the 1990 ESPGHAN criteria for both symptomatic and asymptomatic patients, but 80% do not perform a second biopsy in asymptomatic cases or a gluten challenge in children less than two years of age. Furthermore, most centers (72,5%) do not prescribe a gluten free diet to asymptomatic patients with positive serology and normal bowel architecture (i.e. potential cases), but they do program a careful follow-up.

In conclusion, ESPGHAN criteria are widely followed by Italian CD centers. However their revision might be useful, but it should be evidence-based. Large, multicenter studies are greatly needed.

THE COELIAC DISEASE DIAGNOSTIC APPROACH OF THE ITALIAN PEDIATRICIANS

Renata Auricchio, Viviana Granata, Melissa Borrelli, Riccardo Troncone and on behalf of the Italian Society for Pediatric Gastroenterology Hepatology and Nutrition (SIGENP)

Department of Pediatrics & European Laboratory for the Investigation of Food-Induced Diseases, University Federico II, Naples

Corresponding author:

Prof. Riccardo Troncone
Professor of Pediatrics
Department of Pediatrics and
European Laboratory for the
Investigation of Food-Induced Diseases,
University Federico II
Via Pansini 5, 80131 Naples, Italy
Tel +39 081 7463383
Fax +39 081 5469811

Email address: troncone@unina.it

Introduction

Celiac disease (CD) is defined as a small bowel enteropathy triggered by the ingestion of wheat gliadin and related prolamines in genetically susceptible individuals (1). It is now clear that genetic susceptibility to CD is determined, in particular, by the presence of some alleles of the class II major histocompatibility complex such as DQA1*0501-DQB1*02 (DQ2) and DQA1*0301-DQB1*0302 (DQ8) (2). CD encompasses a large spectrum of clinical manifestations ranging from serious symptomatic forms to completely asymptomatic ones (3). Likewise, histological changes in the small intestinal mucosa can range from typical severe lesions (villous atrophy, hyperplasia of the crypts), to mild forms characterized by minor degrees of enteropathy (4).

The diagnosis of CD normally follows the recommendations formulated by the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). The first diagnostic criteria for CD were defined in 1969 (5). The main requirement was the finding of subtotal villous atrophy in the small bowel mucosa in patients who consumed a normal gluten-containing diet. Further, a clinical and histological improvement on a gluten-free diet and, finally, a recurrence of the typical mucosal lesion after gluten challenge had to be demonstrated by two more biopsies. However, the required deterioration in the histological lesion upon gluten-challenge, and the need for a third biopsy have been a matter of debate. It was suggested that the gluten challenge would only be necessary in atypical or unclear cases.

In 1990, the current diagnostic criteria for childhood celiac disease were introduced by the members of ESPGHAN (6). It was decided that the diagnosis for CD could be based on the finding of mucosal lesion in the small bowel biopsy specimen and full clinical remission after withdrawal of gluten from the diet. The finding of serum anti-gliadin (AGA) and antiendomysium (EMA) antibodies and their disappearance on a gluten-free diet, together with the immunohistochemical analysis (particularly an increased infiltration of intraepithelial

lymphocytes) were considered as supporting evidence for the diagnosis of CD (6). Under the 1990 guidelines, a control biopsy to verify the consequences of the gluten-free diet on the mucosal architecture is considered mandatory just in those patients with equivocal response to the diet, and in asymptomatic patients at first presentation. Further, gluten challenge is encouraged only when there are doubts regarding the initial diagnosis or the adequacy of the clinical response to a gluten-free diet. In children under 2 years of age, the guidelines state that a gluten challenge may be advisable, preceded and followed by a small bowel biopsy.

Since the introduction of the revised ESPGAN criteria in 1990, serological methods and genetic analysis have greatly improved with the introduction of anti-tissue transglutaminase antibodies (anti-tTG) measurement (7). Similarly HLA typing has been increasingly used in clinical practice (8). At the same time, the awareness of different clinical manifestations of celiac disease has evolved, as well as the recognition of a wide spectrum of histological alterations. However, the diagnostic criteria have not been re-evaluated since 1990.

The aim of this study is to investigate the diagnostic methods regarding childhood CD in Italy. The study was designed in order to create the basis for a possible revision of the 1990 ESPGHAN guidelines.

Methods

Through the Italian Celiac Association (AIC) and the Section on Food-Related Disorders of the Italian Society for Pediatric Gastroenterology Hepatology and Nutrition (SIGENP) a nationwide questionnaire on current diagnostic routines was sent by mail to 54 Italian centers for CD diagnosis, fairly distributed on the territory. Forty centers (72.2%) responded by email. The questionnaire contained 12 multiple choice questions, divided in three sections: the first section regarded the serological tests used in the centers, the HLA

typing and immunohistochemistry, if performed; the second section was focused on the diagnostic criteria currently used in symptomatic and asymptomatic children; the third section concerned the clinical management in some special cases, such as children below the age of 2 years, children with positive serology and normal mucosal architecture (potential CD), and IgA deficient patients.

Results

Diagnostic tools

In all the centers anti-tTG measurement is routinely performed. 77,5% still use EMA antibodies detection to confirm the results of anti-tTG measurement. 52,5% still use AGA (IgA and IgG) for diagnosis, especially in children younger than 2 years.

87.5% of the centers perform HLA typing; half of them perfom extended molecular HLA typing. In most cases the test is used to ascertain susceptibility to develop the disease, especially in high risk patients, such as first degree relatives, but it is also used in unclear cases mainly to exclude the possibility of CD.

Up to 67.5% of centers consider an infiltrative lesion (Marsh 1) consistent with CD diagnosis; interestingly, 15% will make a diagnosis of CD even with completly normal mucosa, if patients are symptomatic and have a positive serological analysis. 80% of centers perform immunohistochemical analysis in dubious cases; the most frequently analyzed marker (10/12 of centers) is the number of CD3+ intraepithelial lymphocytes (IEL); 8/10 of centers consider it together with one or more immunohistochemical markers (CD4, CD8, CD25); only 5% analyze γδ intraepithelial lymphocytes.

Diagnostic criteria

The majority of centers (85%) use the 1990 ESPGHAN criteria for both symptomatic and asymptomatic patients, while only 20% will perform a second biopsy in asymptomatic patients on gluten free diet, as the current ESPGHAN criteria require, for diagnosis in clinically silent patients. Most of the centers (80%) do not perform gluten challenge in children under the age of 2, again contradicting the current ESPGHAN guidelines.

Management of special cases

The survey revealed that most Italian pediatricians (72,5%) do not prescribe a gluten-free diet to asymptomatic patients with potential CD, but in any case they require their patients to undergo a careful clinical and serological follow-up including new biopsies.

60% of centers always perform a small bowel biopsy when IgA-deficient patients have CD-related symptoms, regardless of the serological results (IgG anti-tTG). In asymptomatic patients, on the contrary, 65% perform a biopsy only after having tested the IgG anti-tTG antibodies levels.

Discussion

We investigated procedures used by Italian pediatricians in their diagnostic approaches to CD. In recent years, with the recognition of minor degrees of enteropathy, jejunal histology is no longer considered to be the most detailed test available. At the same time serological and genetic tests have acquired a greater importance in the CD diagnosis. For these reasons, a new diagnostic approach, based primarly on antibodies and genetics, has been proposed by many.

Serological tests presently available are anti-tTG, EMA and AGA antibody. As shown in our results, anti-tTG are the most frequently used, followed by EMA to confirm the results. In a smaller number of cases, an AGA antibody measurements is still used. It is difficult to

predict if the emergence of the measurement of deamidated gliadin peptide antibodies (9) will change this practice.

In dubious cases, genetic testing is now frequently used to exclude diagnosis in DQ2/DQ8 negative patients, due to its high negative predictive value. The great majority of centers use genetic testing (in particular extended molecular HLA typing) in unclear cases and also in at risk first degree relatives. In the last group of subjects it is now not only possible to assess susceptibility, but also to quantify risk (10).

The interpretation of jejunal histology has evolved significantly: villous atrophy is not always considered necessary to confirm diagnosis, as minor degrees of mucosal damage, such as crypts hyperplasia, or an infiltrative lesion, are also considered to be consistent with the disease. 67,5% of centers consider an infiltrative lesion in intestinal mucosa to be suggestive of CD. In some cases, completely normal mucosa is taken to be consistent with the diagnosis of CD in symptomatic patients with positive serology.

Despite the new diagnostic tools available, ESPGHAN criteria are still widely followed by Italian diagnostic centers; the only exception regards the gluten challenge, which is not performed in children younger than 2 years: only 20% of the centers follows ESPGHAN recommendations for younger patients. Interestingly, more than 80% do not perform a second biopsy to verify the effects of a gluten-free diet in clinically silent patients, as recommended by the 1990 criteria.

The necessity of an intestinal biopsy has now come into question. It is known that patients with high titers of anti-tTG antibodies, HLA DQ2 or DQ8 positivity and gluten-dependent symptoms almost invariably have a villous atrophy (1); in these cases diagnosis could probably be established without small bowel biopsy (11). Moreover, it has been recognized that many patients with normal mucosal architecture suffer from gluten-dependent symptoms, and even CD related complications such as osteoporosis, even before the

development of villous atrophy. These patients with potential or early-developing CD do not fulfill the traditional ESPGHAN diagnostic criteria. Although virtually all centers perform intestinal biopsy, it is clear that jejunal biopsy has a less fundamental role than in the past. On the other hand, the contribution of the analysis of jejunal biopsies may still be very important. The study of the intestinal mucosa could play a decisive role in the definition of the disease. When CD is suspected, but histology is not diagnostic (for example in potential CD or in early-developing celiac disease), increased density of γδ lymphocytes (12) and villous tip IEL (13) supports the diagnosis of CD. Few centers, however, are able to perform such an analysis since it requires frozen biopsies.

In conclusion, many open questions on CD diagnosis still remain. A revision of the current ESPGHAN criteria might be useful, as has already been frequently suggested, but it should be evidence-based. Large, multicenter studies are greatly needed.

Acknowledgments.

We are indebted to all Italian pediatric centers for CD that answered to the questionnaire. We also thank Simona Ruggiero for her editorial assistance.

This study is supported by grants from Regione Campania, Assessorato alla Ricerca, (fondi ex legge 5) and Assessorato alla Sanità (Progetto Rete Regionale per Bambini e Adolescenti affetti da Malattia Celiaca).

References

1. Green PH, Jabri B. Coeliac disease. *Lancet* 2003;362:1418-9.
2. Babron MC, Nilsson S, Adamovic S, Naluai AT, Wahlström J, Ascher H, Ciclitira PJ, Sollid LM, Partanen J, Greco L, Clerget-Darpoux F; European Genetics Cluster on

Coeliac Disease.Meta and pooled analysis of European coeliac disease data. *Eur J Hum Genet.* 2003;11(11):828-34

3. Ravikumara M, Tuthill DP, Jenkins HR. The changing clinical presentation of coeliac disease. *Arch Dis Child.* 2006;91(12):969-71.
4. Marsh MN. Gluten, major histocompatibility complex, and small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity. *Gastroenterology* 1992;102:330-354.
5. Meuwisse GW. Diagnostic criteria in celiac disease. *Acta Paediatr Scand* 1970;59:461.
6. Walker-Smith JA, Guandalini S, Schmitz J, et el. Revised criteria for the diagnosis of celiac disease. *Arch Dis Child* 1990;65:909-11.
7. Troncone R, Maurano F, Rossi M, Micillo M, Greco L, Auricchio R, Salerno G, Salvatore F, Sacchetti L. IgA antibodies to tissue transglutaminase: An effective diagnostic test for celiac disease. *J Pediatr*. 1999;134(2):166-71.
8. Sacchetti L, Tinto N, Calcagno G, Improta P, Salvatore F. Multiplex PCR typing of the three most frequent HLA alleles in celiac disease. *Clin Chim Acta.* 2001 20;310(2):205-7.
9. Sugai E, Vázquez H, Nachman F, Moreno ML, Mazure R, Smecuol E, Niveloni S, Cabanne A, Kogan Z, Gómez JC, Mauriño E, Bai JC. Accuracy of testing for antibodies to synthetic gliadin-related peptides in celiac disease. *Clin Gastroenterol Hepatol.* 2006;4(9):1112-7.
10. Bourgey M, Calcagno G, Tinto N, Gennarelli D, Margaritte-Jeannin P, Greco L, Limongelli MG, Esposito O, Marano C, Troncone R, Spampanato A, Clerget-Darpoux F, Sacchetti L.HLA related genetic risk for coeliac disease. *Gut.* 2007;56(8):1054-9.

11. Kaukinen K, Maki M, Partanen J, Sievanen H, Collin P. Celiac disease without villous atrophy: revision of criteria called for. *Dig Dis Sci* 2001; 46:879-88.
12. Spencer J, Isaacson PG, MacDonald TT, et al. Gamma/delta cells and the diagnosis of celiac disease. *Clin Exp Immunol* 1991;85:109-113.
13. Jarvinen TT, Collin P, Rasmussen M, Kyronpalo S, Maki M, Partanen J, Reunala T, Kaukinen K. Villous tip intraepithelial lymphocytes as markers of early-stage celiac disease. *Scand J Gastroenterol.* 2004;39:428-33.

2. SPECTRUM OF COELIAC DISEASE: "POTENTIAL" COELIAC DISEASE.

The second line of my research had the aim of elucidation of natural history of children with serum positive CD-related antibodies and architecturally normal small intestinal mucosa.

Introduction

In the last decade, it has become increasingly evident that CD is not restricted to severe gluten-dependent enteropathy. From an histological point of view, in fact, CD represents a spectrum that goes from the typical severe lesions of duodenal mucosa, such as villous atrophy, to forms with a normal architecture of duodenal mucosa and only an increased infiltration of the intraephitelial lymphocytes (IELs) (Marsh MN 1992). In clinical practice, by increased diagnostic efficiency of serum CD-related autoantibodies measurements and increased attention for CD than in the past, it is increasingly more common to find patients who have serum positivity for CD autoantibodies but whose duodenal mucosa shows a normal histological appearance. The potential CD is a patient with anti-endomysium antibodies (EMA) and/or anti-human tissue transglutaminase antibodies (anti-TG2) in serum, but a normal mucosal architecture at the intestinal biopsy examination. Good markers of potential CD include subtle pathological alteration such as increased density of intraepithelial

lymphocytes expressing γδ TCR, signs of activated mucosal cell-mediated immunity (such as expression of CD25 and B7 by lamina propria mononuclear cells), coeliac-like intestinal antibody pattern. Recent work have demonstrated that first-degree relatives of coeliac patients presents a positive rectal gluten challenge (Troncone R 1996, Paparo F 2005). Clinical, histological and serological markers predictive of atrophy developing are not known.

The IELs are increased in the mucosa of untreated coeliac patients, but these cells are not pathognomic for CD. Certainly, an increase of δγ + IELs (Jarvinen TT 2003) or the count of intraepithelial lymphocytes at the villous tip (Jarvinen TT 2004) strengthens the probability of CD in borderline cases where the histology is difficult to interpret. Finally, in a recent study with the aim to identify a marker to detect coeliac disease before the development of villous atrophy, the authors demonstrate that these deposits would have the best sensitivity and specificity to predict the evolution to overt CD (Salmi TT 2006).

The clinical spectrum of potential CD, like the classical coeliac disease, is wide. The patients with serum CD-related antibodies and intact duodenal villi can present at diagnosis intestinal complaints, such as abdominal pain, diarrhoea, nausea, vomiting, and bloating or have extraintestinal symptoms or be silent, as described in a recent study (Dickey W 2005). Dickey W et al report a incidence of potential CD of 10% in adult population: the 34%

of potential CD patients were first-degree relatives and the 17% were affected by autoimmune disease.

The only accepted treatment for CD is a strict adherence to gluten-free diet (GFD), on which histology and symptoms should normalize (Walker-Smith JA 1990). There are no clear guidelines as to how best to deal with potential CD subjects, and in particularly whether a life-long gluten free diet (GFD) is necessary in subgroup of asymptomatic patients with normal intestinal mucosa and serum CD-associated antibodies. It is quite clear that subjects with severe gluten dependent enteropathy face a series of health risks, mainly nutritional; they probably have also higher risk of developing autoimmunity, and, although less than previously thought, of presenting neoplastic complications. On the contrary, little is known of those with minor enteropathy. A recent report (Kaukinen K 2001) showed nutritional deficiencies also in patients with minor enteropathy, positive serology and "risk" genetics, resolving on a gluten free diet.

Starting from consideration that more information is also needed on the natural history of patients with minor inflammation, in particular on features influencing the degree of gluten-dependent inflammation, on the markers predictive of evolution to frank CD and on the health risks they are exposed to, if left on a normal gluten-containing diet, the objective of my research was the characterization of clinical, biological and histological

features of children with positive CD-associated serum antibodies and architecturally normal small intestinal mucosa by longitudinal evaluation.

Patients

At this propose our study involved 80 children (24 male and 56 female with a median age of 6 years and 8 month; range 18 months-16 years and 2 months), who underwent small intestinal biopsy for the suspicion of CD in the Department of Pediatrics in Naples University Hospital "Federico II" Italy from January 2000 to December 2007. They were selected according to the presence in their serum of EMA and/or a value of anti-TG2 antibodies higher than cut-off and architecturally normal small intestinal mucosa (Marsh 0 and Marsh 1). We have excluded patients with IgA deficiency.

These potential CD patients were strictly followed-up every sex months with: clinical evaluation (symptoms evolution, growth parameters, amount of daily gluten intake,) laboratory evaluation (CD-related autoantibodies, thyroid autoantibodies, nutritional parameters, organ functionality, bowel inflammatory indexes). A jejunal biopsy was performed again every two years of follow-up in patients with persistent positivity for EMA and/or anti-TG2; before, only in case of symptoms development.

Results

Patients with potential coeliac disease represented the 14.1% (194/1373) of the patients with diagnosis of coeliac disease in the considered period.

Patients characteristics

35/80 patients belonged to "at risk group"; 22 were first-degree relatives of coeliac patients and 13 presented other autoimmune diseases: 11 were diabetics patients and 2 had a thyroiditis. Most of them (43/80 53.7%) were asymptomatic while 37/80 presented symptoms, in particular 33 gastrointestinal symptoms (e.i. recurrent abdominal pain, weight loss, recurrent diarrhea, failure to thrive); and 4 extraintestinal symptoms (e.i. short stature, iron deficiency anemia). The decision of beginning a GFD was based no symptoms severity.

Patients that began a GFD

16/80 patients began a gluten free-diet because of symptoms or signs suggestive of CD: 2 for short stature with no other causes; 2 for pathological value of Z-score at BMD; 3 for failure to thrive; 5 for recurrent abdominal pain; 2 for diarrhoea; 1 for thyroditis and dilatative cardiomyopathy. In 1 case the parents refused to start GFD: the indication was due to a pathological value of Z-score at BMD. 6/15 didn't show any response to GFD: 2 continued to have the same stature percentile of the beginning after more than 2 years of diet, 2 continued to present a failure to thrive after 1 year of diet and in 2 cases the recurrent abdominal pain

persisted. In 3/6 cases, for the absence of response, the patients started again a gluten containing diet and repeated a second biopsy that resulted normal. In the 2 cases in which the diet was begun for a pathological value of Z-score at BMD we have not repeat the exam yet because less than 2 years have passed. All patients on a GFD presented negativization of CD-related serology as any other coeliac patient with atrophy (Tab. 1).

Patients that continued a gluten containing diet

The 64/80 patients that continued a gluten containing diet and the 3/15 that started a gluten containing diet again underwent a strictly follow-up every-six months. All the asymptomatic patients continued to be asymptomatic while in 11 cases symptoms presented at the moment of the diagnosis disappeared during the follow-up (Tab.1).

Autoimmune diseases

Of the 67 patients that continued a gluten free diet 1/67 developed a type 1 Diabetes few months after the first biopsy and 3/67 developed a thyroiditis, in 1/3 the patients became negative for CD-related antibodies before the developing of the thyroiditis.

Dietary assessment

At the moment of the first biopsy the daily gluten intake of patients with potential CD had a media of 15 grams with a range from 6 to 40 g/die. These values were equivalent to those of control group. Even the calcium quantities were similar to those of the control group.

HLA typing

In 67/80 HLA typization was performed. 66/67 patients presented an HLA consistent with CD with a positivity of DQ2 and/or DQ8: 46/67 were positive for HLA A1*0501 B1*0201—two haplotype either in *cis* or in *trans*, and 10/67 for the HLAB1*0302 haplotype; 5/67 patients presented a positivity for HLA A1*0501 B1*0201 and HLAB1*0302, while only 5/67 presented a positivity only for HLA A1*0501 or HLA B1*0201. Only 1 patients was negative for all the HLA haplotypes associated with CD, his HLA was DQB1*05/DQB1*06.

Serology

At the moment of the first biopsy 64/80 patients were positive for both EMA and anti-TG2, 14/80 patients only for EMA and 2/80 patients only for anti-TG2. Anti-TG2 titres were significantly lower than those of coeliac patients with atrophy as already demonstrated in a previous work (Paparo F 2005).

During the follow-up 32/67 patients that continued a gluten containing diet had a persistently positive serology with EMA positivity and values of anti-TG2 higher than cut-off, in 5/67 cases the serology became completely and persistently negative both for EMA and anti-TG2 while 30/67 showed a fluctuation of antibodies titres with transient negativity for EMA or anti-TG2 values. The fluctuation and negativization were more frequent in those who at the beginning presented lower titres of anti-TG2.

Nutritional Parameters

3/67 presented hypoferritinemia that solved with iron therapy, other nutritional parameters and coagulation parameters were normal in everyone.

Bowel inflammatory indexes

To evaluate the bowel inflammation we performed fecal calprotectin and permeability test in 49/80 and 22/80 patients respectively, and we found that 10/49 presented a positive value of fecal calprotectin while 3/22 showed an altered result of permeability test. We repeated the test in 17/49 and 9/22 and we found in 4/17 and in 1/9 pathological values respectively.

Computerised bone mineralometry

At the moment of the first biopsy 39/80 patients underwent a computerised bone mineralometry study and 3/39 presented Z-score values compatible with an osteoporosis, 2 of them began a GFD while another continued a gluten containing diet for parental decision. We repeated the exam in 7/39 and all were normal after 2 years of follow-up.

Histology, immunohistochemical analysis

All patients presented an intestinal mucosa normal from an architecturally point of view, 36 patients were M0 and 44 M1. 75 patients underwent an immunohistochemical analysis. 44/75 (58.6%) patients presented a value of CD3+ cells higher than cut-off and 53/75 (70.6%) an increased value of γδ+ cells. Even in the lamina propria 49/75 (65.3%) patients presented a number of CD25+ cells higher than cut-off (Tab.2).

Second biopsy

29/67 patients that continued a gluten containing diet underwent a second biopsy after two years of follow-up. In 28/29 for the positivity for EMA and/or elevated values of anti-TG2 antibodies and only in one case the biopsy was performed because of invalidating abdominal pain even without a positive serology. 7/29 developed an intestinal atrophy receiving diagnosis of CD and beginning a GFD. In 22/29 the mucosa continued to be normal and 6/22 were M0 and 16/22 were M1 (Tab. 1). In 21/22 patients with persistently normal intestinal mucosa we performed an immunoistochemical analysis; 15/21 (71.4%) patients presented a value of CD3+ cells higher than cut-off not statistically different from patient with normal mucosa at the moment of the first biopsy; 20/21 (95.2%) presented an increased value of γδ+ cells, in this case there was a difference statistically significant from the time of the first biopsy (Tab 2). In the lamina propria 11/21 (52.3%) patients presented a number of CD25+ cells higher than cut-off. Only one patient had already performed a third biopsy that was still normal.

Deposits

41/80 patients were evaluated for the presence of intestinal deposition of IgA anti-TG2 and 27/41 (65.8%) were positive with a concordance index of 78.0% (32/41) with the positivity of serum anti-TG2, the values of anti-TG2 in patients without deposits were statistically lower than those of patients

posits for intestinal deposits ($p < 0.05$); in most cases they presented a patchy distribution of deposits with tracts of clear positivity and tracts with absent signal. 29/29 patients that underwent a second biopsy were analyzed for intestinal deposits and we found that 21/29 (72.4%) of them were positive for intestinal deposits, 7/21 presented atrophic mucosa. The number of positive patients had no statistically significant increase .

Searching for markers predictive of atrophy developing

We compared with a Pearson's Chi-Square test different parameters to find markers that at the moment of the diagnosis could help us to identify those patients who had the risk to develop an atrophy. The only parameter that was near to significant value was the belonging to "at risk group"(Tab. 3).

Discussion

The aim of our work was to evaluate the natural history of patients with positive serology for CD but with a normal intestinal mucosa, the so-called potential coeliac patients. This kind of patients is increasing, perhaps because of the screening of population "at risk" or because nowadays the attention for CD is higher than in the past. The percentage of these patients is now near 20% of patients with positive serology that undergo a small intestinal biopsy. All the patients evaluated for HLA except for one

presented an HLA compatible with CD so we could think that the probabilities that positive serology is a laboratory mistake is very low.

Some works in literature state that this absence of intestinal damage could be connected with a low amount of gluten in the diet, for example in first degree relatives of coeliac patients (Maki M 1992; Valletta E 2002); our data showed that these patients had a daily gluten intake equivalent to those of control patients matched for age and sex.

These patients are often asymptomatic or with light symptoms that are sometimes transient and that in some cases solve even on a free diet; the symptomatic patients began a gluten free diet but not always there was a gluten-dependence of their symptoms. As concerns patients that had continued a gluten containing diet we found that there was no significant alteration of the principle nutritional parameters during the follow-up.

At the moment of first biopsy the anti-TG2 titres was lower than that showed by coeliac patients with atrophy as already showed in a previous work (Paparo 2005), except for a few number of patients. The majority of them during the follow-up became negative or presented fluctuant values of anti-TG2 or EMA. These cases were more frequent among those who at the beginning had a lower titre of anti-TG2.

As concerns the histological and immunoistochemical parameters we confirmed that in the majority of patients there are signs of cells activation

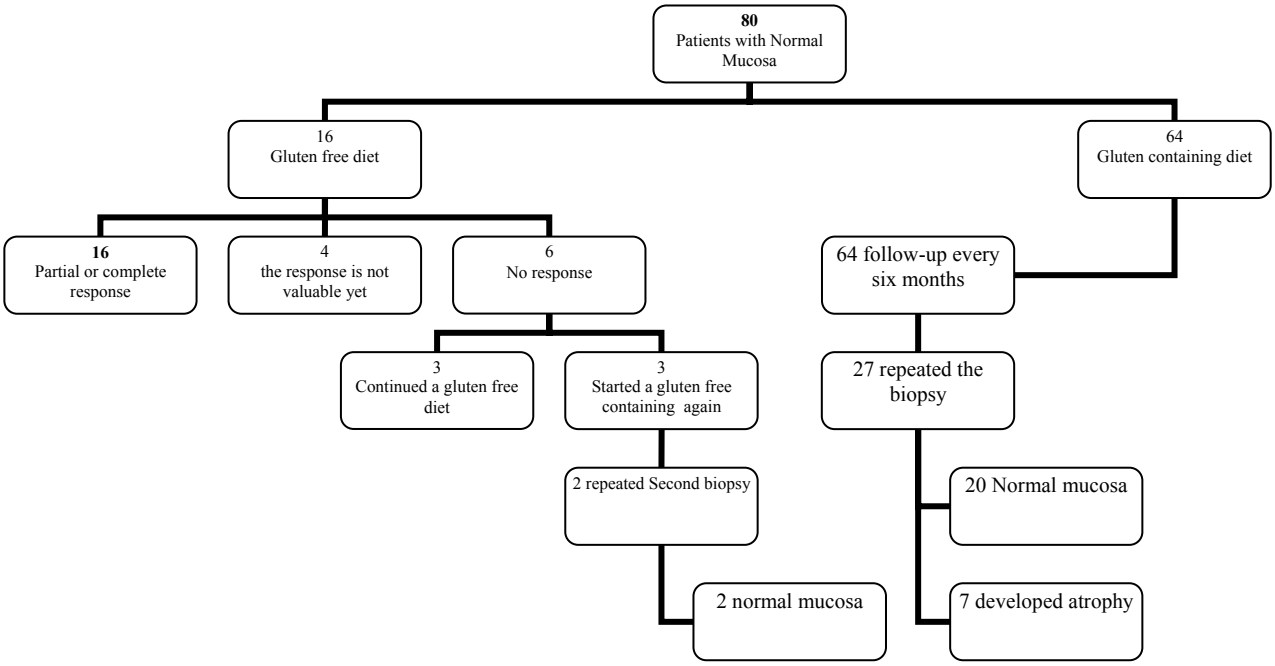
with increased values of CD3+ positive cells and above all gamma/delta+ cells higher than cut-off as already showed (Paparo et al 2005)

The markers predictive of evolution to frank atrophy remain to be assessed. In particular data in literature show that intestinal deposits could be present at a mucosal level before the appearing of anti-TG2 in serum (Kaukinen K 2005). So that their detection could be the most sensitive and specific test for diagnosis of CD. Our work showed that there is a 34.1% of patients that are negative for intestinal deposits of IgA anti-TG2 even in presence of serum EMA and/or anti-TG2; in particular not always there is a concordance between the presence of intestinal deposits and serum anti-TG2 titers higher than cut-off. Anyway there was no difference in the positivity of intestinal deposits between those patients who developed atrophy and those who continued to have a normal mucosa after 2 years of follow-up.

In literature there is no accordance on which is the better diagnostic and therapeutic protocol for patients with potential CD. There is no doubt that some subjects develop an intestinal atrophy but there is even a 7.4% that become persistently and completely negative.

The data, obtained by this study, are very interesting, but they are not conclusive. A prospective and randomized study with longer follow-up is necessary.

Tab.1 Summary of patients and dietary assessment



Tab. 2 Immunohistochimical analysis in patients with normal intestinal architecture.

Immunoistochemical parameters	First biopsy N=75	Second biopsy N=21	*P value*
CD3+ cells (> 35 mm/epithelium)	44/75 (58.6%)	15/21 (71.4%)	*P =0.2*
γδ+ cells (> 3.2 mm/epithelium)	53/75 (70.6%)	20/21(95.2%)	***P=0.019***
CD 25 + cells (4 cells / mm^2 lamina propria)	49/75 (65.3%)	11/21(52.3%)	*P=0.27*

Tab.3 Pearson's Chi-Square test between different parameters.

Parameters at the time of first biopsy	CD potential patients who developed atrophy N°7	CD potential patients who continued to have Normal Mucosa N°22	*P value*
Familiarity	4 (57.1%)	7 (31.8%)	*P =0.22*
Autoimmunity	2 (28.5%)	5 (22.7%)	*P=0.75*
Familiarity+autoimmunity	6 (85.7%)	11 (50%)	*P=0.09*
Marsh 1	4 (57.1%)	14 (63.6%)	*P =0.75*
Increased value of γδ+ cells	5 (71.4%)	17(77.2)	*P=0.75*
Serology persistently positive	5 (71.4%)	14 (63.6%)	*P=0.70*
Presence of intestinal deposits	6 (85.7%)	15 (68.2%)	*P=0.36*

3. IMMUNOLOGIC MARKERS IN POTENTIAL COELIAC DISEASE: CYTOKINES AND T REGULATORY CELLS

After a study of clinical, biological and histological features of potential CD, the aim of my research was to characterized the immune respose in the intestine of patients with potential CD, in particular to clarify the early immunological events leading to enterophaty in the small intestine.

Introduction

Evidence suggests that coeliac disease develops gradually from small bowel mucosal inflammation to crypt hyperplasia and eventually to overt villous atrophy. The immunological mechanisms that in the potential coeliac mucosa are able to prevent the progression towards a complete mucosal damage are not yet known. Very likely, there is an interplay between an adaptive immunity characterised by a specific and memory T cell response, innate immunity involving less specific mechanisms and immune regulation mediated by IL-10.

There is strong evidence that CD4+ T cell-mediated hypersensitivity plays a major role in tissue injury. Lamina propria CD4+ T cells are phenotypically activated and produce large amounts of Th1 cytokines in response to gluten stimulation (Nilsen EM 1998, Breese EJ 1994). IFN-γ seems to play a central role. IFN- γ production is massively increased in

untreated coeliac mucosa and *in vitro* its secretion can be observed in organ cultures from treated CD patients and in lamina propria derived T cell clones upon stimulation with gliadin peptides (Nilsen EM 1998, Troncone R 1998). Many important questions remain however regarding factors which induce and maintain Th1 cell polarisation in CD. Paradoxically, IL12, the major Th1 inducing cytokine, is undetectable in the mucosa of active CD (Nilsen EM 1998, Monteleone G 2001). IFNα, another cytokine able to promote Th1 differentiation and IFNγ production in humans, is expressed in the mucosa of untreated CD (Monteleone G 2001). In addition, it was showed the occurrence of coeliac disease in patients receiving IFNα therapy, suggesting a role for this cytokine in promoting the local immune response in CD (Monteleone G 2001). Furthermore, in CD but not in normal duodenal mucosa, there is production of active IL18, a cytokine with crucial role in maintaining Th1 response in human disease and experimental models (Salvati VM 2002). Finally, it has recently been proposed that IL15, a cytokine that is able to expand Th1 cell differentiation under particular conditions, may be involved in the CD immune response (Maiuri L 2000, Seder RA 1996).

However, the transcriptional mechanism that underlie the distinct Th1 type cytokine repertoire in CD remain unknow. A crucial transcription factor involved in the early phase of Th1 differentiation is T-bet, a novel member of the T-box family of transcription factors. T-bet drives chromatic

remodelling of the IFNγ locus. T-bet RNA transcripts and proteins are enhanced in biopsies from untreated CD patients (such as in Crohn's disease). The factors which induce T-bet in CD remain to be determined but some observations underline the relevance of the STAT-1 signalling pathway (Monteleone I 2004). Furthermore, STAT-1 and Interferon Regulatory Factor(IRF)-1, the transcription factors downstream the signalling pathway elicited by IFN-γ, are activated in untreated coeliac mucosa and can be induced *in vitro* in organ cultures from treated CD patients upon stimulation with gliadin peptides and this effect is prevented by a neutralizing IFNγ antibody (Salvati VM 2003). Anti sense oligonucelotides able to block STAT-1 signaling pathway prevent *in vitro* in organ cultures from treated CD patients IFNγ induced gene production of ICAM-1 and B7-1. The positive feedback regulation between IFNγ/STAT-1/IRF-1 is one of the most important mechanism that maintain autoimmunity in other Th1 mediated immune disease (Suk K 2001, Nakazawa T 2001). The molecular mechanism that underlies the ability of IRF1 to promote Th1 cell polarization is not fully understood. It is unlikely that IRF-1 directly affects transcription of the IFNγ gene, because no binding sites for IRF-1 have been reported in the promoter of the IFNγ gene. The more plausible explanation is that IRF-1 facilitates indirectly the induction of Th1 cells by positively regulating the synthesis of Th1-inducing cytokines, such as IFNα, IL15 and IL18 (Fujita T 1989, Fantuzzi

G 2001). It would also be important to emphasize that these cytokines can, through the induction of IFNγ, eventually enhance transcription of IRF-1 and generate a positive feedback loop able to maintain and expand the Th1 response in CD. In addition, IRF-1 can contribute to amplify and maintain chronic inflammation by its ability to modulate several immunoregulatory genes, as well as to facilitate the recruitment of inflammatory cells within the inflamed tissue up-regulating the expression of vascular cell adhesion molecule-1 (Taniguchi T 2001, Kamijo R 1994, Kimura T 1996).

Recently, several studies have suggested that immunosuppressive cytokines such as IL-10 and TGF-β, have an important role in maintaining of intestinal tolerance. In particular, mice which are genetically deficient for IL-10 develop a severe form of enterocolitis, similar to human inflammatory bowel disease (IBD) (Kuhn R 1993), and have increased susceptibility to autoimmune diseases such as rheumatoid arthritis (Samoilova EB 1998) and experimental autoimmune encephalomyelitis (EAE) (Bettelli E 1998). On the other hand, colitis, which develops in SCID mice post transfer of CD4+CD45RB^{high} T cells, can be prevented by IL10 (Powrie F 1994). Generally considered as Th2 cytokine, IL-10 has a crucial role in the differentiation of the subset of CD4+ T regulatory cells, known as Tr1 cells (Roncarolo MG 2001).

In CD the levels of IL10 are higher in untreated CD patients if compared to treated CD patients and controls, but the ratio IL-10/IFNγ is significantly lower.

To better understand the onset of CD we focused our interest on potential CD this condition represent a good *in vivo* model to know the immunological events that prevent the progression towards a complete mucosal damage.

Two different approaches have been designed to achieve this aim:

a. Analysis of cytokines and transcription factor involved in T cell activation (IL-2), Th1 differentiation (IFN-γ, T-bet, IRF-1) and immune regulation (IL10) in potential CD by real-time PCR
b. Analysis of T regulatory cells (CD4+CD25+FOXP3+, CD4+IL10+IL4- and CD4+ IL10+IL4+ Th2 cells) in potential CD by flow cytrometric.

A. Analysis of cytokines and transcription factor involved in T cell activation (IL-2), Th1 differentiation (IFN-γ, T-bet, IRF-1) and immune regulation (IL10) in potential CD by real-time PCR

The first objective of this line of my research was characterized early immunological markers in potential CD by analysis of genes with crucial role in T cell activation (IL-2), Th1 differentiation (IFN-γ, T-bet, IRF-1) and immune regulation (IL10).

Patients

For this aim, by real time PCR analysis we studied duodenal biopsies from: 16 controls (mean age: 5,3 yrs; range: 1.7-13.9) 17 untreated (mean age: 17.6 yrs; range: 0.75-13.3) and 15 potential coeliac patients (mean age: 7.2 yrs; range: 0.83-13.75).

Control patients underwent upper gastrointestinal endoscopy because of the presence of gastrointestinal symptoms (abdominal pain, gastroesophagel reflux, emesis, failure to thrive). Duodenal mucosa was histologically normal in all of them. In 9/16 patients immunohistochemistry was performed. One patient presented an increase of intraepithelial lymphocytes (42 cells/100 enterocytes) and in 5 controls an increased expression of CD25+ lamina propria mononuclear cells was observed (range 4-14 cells/mm^2 lp).

Potential coeliac patients were characterised by the presence of coeliac specific autoantibodies (EMA and anti TG2), the presence of HLA DQ2 and/or DQ8, but a histologically normal mucosa (Marsh 0-1). Two patients were first degree relatives of coeliac patients, 9 patients were investigated for coeliac disease because of abdominal pain, short stature, diarrhea and 4 patients were detected by screening. In all potential coeliac patients immunohistochemistry was peformed. The presence of more than 35 intraepithelial lymphocytes was defined as Marsh 1 lesion. In 9/15 patients a Marsh 1 lesion was observed. In all of them signs of T cell activation such as an increase of CD25+ and ICAM-1 expression in the lamina propria and HLA DR in the crypts were also observed. Particularly, in 6 of these 9 patients we could observe an increase of both TCR γδ + T cells in the epithelium and CD25+ cells in the lamina propria.

In 6 potential coeliac patients a Marsh 0 lesion was reported. In 3 of these patients signs of T cell activation were reported. Only in one patient we observed an increase of both epithelial TCR γδ + T cells and lamina propria CD25 + cells.

Untreated coeliac disease was diagnosed in accord to ESPGHAN criteria (Working Group of ESPGAN 1990).

Results

IL-2 RNA expression is increased in duodenal mucosa from potential CD patients.

We analysed duodenal mucosa from 11 controls, 11 potential coeliac and 12 untreated coeliac patients for IL-2 RNA expression by real-time PCR. We observed a significant increase for IL-2 RNA expression in potential coeliac mucosa if compared to control ($p<0.02$) and to untreated coeliac patients ($p<0.004$) (Fig. 1). We didn't observe any difference between untreated coeliacs and control patients.

Th1 differentiation markers are expressed in duodenal mucosa from potential CD patients

We analysed expression of T-bet, IRF-1 and IFNγ in duodenal biopsies from 16 controls, 15 potential coeliac patients and 17 untreated coeliac patients.

For T-bet RNA expression we could observe a significant increase in untreated coeliac mucosa if compared to control mucosa ($p<0.02$). In potential coeliac patients because of the wide confidence interval means were not statistically different neither compared to control patients nor to untreated coeliacs (Fig. 2).

Similarly, we observed for IRF-1 RNA expression a significant increase in untreated coeliacs if compared to control patients (<0,003). Interestingly,

in potential coeliac mucosa IRF-1 RNA levels were significantly lower if compared to untreated CD ($p<0.02$) (Fig. 3).

We found a significant increase of IFNγ RNA expression in potential CD if compared to control patients ($p<0.0001$) (Fig. 4). Interestingly, IFNγ expression in potential coeliac mucosa correlated significantly with intraepithelial lymphocyte infiltration ($p<0.008$, Pearson $r=0.64$ (Fig. 5). To support this observation we found that in potential CD patients with Marsh 1 lesion IFN-γ expression was significantly over-expressed if compared to potential CD patients with Marsh 0 and controls ($p<0.0001$). In Marsh 0, instead, IFN-γ levels did not differ significantly from control patients ($p=0.178$). Remarkably, even if increased in potential CD mucosa IFN-γ levels were still significantly down-regulated if compared to untreated CD ($p<0.0001$) (Fig. 4).

IL-10/IFNγ ratio is increased in duodenal mucosa from potential Marsh 0 CD patients

We analysed IL-10 RNA expression in 16 controls, 15 potential CD (9 Marsh 1 and 6 Marsh 0) and 17 untreated CD patients.

In contrast to IFN-γ RNA expression we observed for IL-10 RNA expression a significant up-regulation in Marsh 0 CD patients if compared to Marsh 1 lesion ($p<0.005$) and control patients ($p<0.001$) (Fig. 6). The inverse correlation between IL-10 and IFN-γ RNA expression was even

more striking and became significant in untreated CD patients (Pearson r=-0,62 <0,007). As a consequence when we analysed the ratio between IL-10 and IFN-γ RNA levels we observed that in Marsh 0 potential CD patients the ratio was significantly over-expressed if compared to potential CD patients with Marsh 1 lesion ($p<0.001$), to untreated CD ($p<0.0001$) and even to control patients ($p=0.053$). In untreated CD patients the ratio was significantly down-regulated if compared to control patients ($p<0.001$) (Fig. 7).

B. Analysis of T regulatory cells (CD4+CD25+FOXP3+, CD4+IL10+IL4- and CD4+ IL10+IL4+ Th2 cells) in potential CD by flow cytrometric.

On the basis of the preceding data, showed that in the early phase of the potential CD (Marsh 0) T cells are activated and committed to Th1 and the ratio between was increased significantly than Marsh 1 lesion and untreated CD; we suppose that regulatory mechanisms might down-regulate T cell mediate immune response.

Thus, the second objective of this line of my research was to analyse in the different stage of coeliac enteropathy the type and percentage of regulatory T cell populations mainly CD4+CD25+FOXP3+ regulatory cells, CD4+IL10+IL4- (known as Tr1) and CD4+IL10+IL4+ Th2 cells.

Patients

For Intracellular Cytokine Detection by Flow Cytometry, 18 controls (mean age: 10.5 yrs; range: 1.3-17.6) 26 untreated (mean age: 6.7 yrs; range: 1-17.8) and 27 potential coeliac patients (mean age: 10 yrs; range: 2-17.3) were recruited.

Control patients underwent upper gastrointestinal endoscopy because of the presence of gastrointestinal symptoms (abdominal pain, gastroesophagel reflux, vomiting). Two patients were affected by colonic inflammatory bowel disease. Four patients were Helicobacter pylori positive. Duodenal mucosa was histologically normal in all of them. In 3/18 patients immunohistochemistry was performed. No patient presented an increase of intraepithelial lymphocytes or of CD25 lamina propria mononuclear cells. Only in one patient an increase of density of cells expressing TCR γδ+ in the intraepithelial compartment (27 cells/ mm epithelium) was observed.

Potential coeliac patients were defined as mentioned above. Six patients were first degree relatives of coeliac patients, nine asymptomatic patients were detected by screening (seven patients were affected by insulin dependent diabetes mellitus (IDDM) and two by thyroiditis), the others were investigated for coeliac disease because of abdominal pain, diarrhea, growth failure, weight loss, vomiting and anemia. In 18/27 potential CD patients a Marsh 1 lesion was observed. In 15 of them

immunohistochemistry was peformed and revealed in 13 patients the presence of immunological features of T cell activation such as an increase of CD25+ cells, TCR γδ+ intraepithelial lymphocytes, ICAM-1 expression in the lamina propria and HLA DR in the crypts. In 9/27 potential coeliac patients a Marsh 0 lesion was reported; in 4 of them we observed an increase of both TCR γδ+ intraepithelial lymphocytes and CD25+ cells in the lamina propria, while in 6 only an increase of TCR γδ+ intraepithelial lymphocytes and in 5 only an increase of CD25+ lamina propria mononuclear cells were reported.

Results

IL-10 in duodenal coeliac mucosa derives from T cells

In accord to RNA expression we revealed by FACS analysis a significant increase of the percentage of IL-10 producing cells in the mucosa of potential coeliac patients ($p<0.003$) compared to control patients. We also observed an increased percentage of IL-10 producing cells in untreated coeliac patients ($p<0,005$).

In order to investigate the role of IL-10 we characterised by FACS analysis the phenotype of IL-10 producing cells. We performed three-colour staining with CD56, CD3 and IL-10. We analysed in the gate of IL-10 positive cells the percentage of CD3+ CD56- (T cells), CD3- CD56+ (NK) and CD3+ CD56+ (NKT cells) which are the main producers of IL-

10 in the mucosa. We could observe that in all groups of patients the mean percentage of IL-10 positive cells was significantly higher in the CD3+ CD56- population (Fig. 8).

Increased percentage of IL10+ cells expressing CD4 surface marker in the duodenal mucosa from Marsh 0 potential coeliac patients

Since different populations of T cells able to counteract Th1 immune response such as Tr1 cells (CD4+ IL10+ IL4-), Tregs (CD4+CD25+FoxP3+) and Th2 cells (CD4+IL10+IL4+) in the intestinal mucosa express CD4 surface antigen we analysed by double staining the percentage of IL10+ CD4+ cells.

Interestingly, we observed that the percentage of CD4+ IL10+ T cells was significantly increased in Marsh 0 if compared to control patients ($p<0,03$) and to untreated CD patients ($p<0,001$) and was near to significance if compared to Marsh 1 lesion ($p=0,07$) (Fig. 9) suggesting the presence of immunoregulatory mechanisms in Marsh 0 potential CD which is also supported by the high IL-10/IFN-γ RNA ratio mentioned above.

In untreated CD the percentage of CD4+IL10+ cells was significantly down-regulated if compared to control ($p<0,004$), Marsh 1 ($p<0,04$) and Marsh 0 patients ($p<0,001$).

Increased percentage of CD4+CD25+FoxP3+ cells in duodenal mucosa from coeliac patients

To characterise the phenotype of CD4+ cells we performed three-colour staining for IL10/CD4/IL4 and CD4/CD25/FoxP3.

The percentage of IL-10 positive cells that express CD4 and IL4 (Th2) was at a first glance not significantly different in all groups analysed. However, when we splitted the control group in normal controls and Helicobacter pylori positive controls we could observe a significant increase of the percentage of Th2 cells in Marsh 0 and untreated CD patients if compared to control patients ($p<0,03$ and $p<0,05$ respectively) (Fig. 10).

We couldn't observe any difference in the percentage of IL-10 positive cells that express CD4 but not IL4 (Tr1) (Fig. 11).

Interestingly, a significant increase was observed in untreated CD, Marsh 0 and Marsh 1 potential CD mucosa for CD4+ cells that expressed CD25 and FoxP3 if compared to control mucosa ($p<0,0001$, $p<0,0028$ and $p<0,0008$ respectively) (Fig. 12).

Discussion

In this line of my research, for the first time, we focused our interest on potential CD and, in particular, on their intestinal immune response,

representing this condition a good *in vivo* model to know the early immunological events implicated in the onset of coeliac disease.

The first object of this study was to analyse the state of activation of mucosa T cell in potential CD. At this aim, we studied the RNA expression for IL-2 by quantitative RT-PCR. IL2 is an important growth factor for T lymphocytes and is involved in T cell activation and differentiation (Jain J 1995). We observed a increase of IL-2 RNA expression in potential CD patients if compared to controls and untreated coeliacs, while there were not any difference between untreated CD and control patients, in accord to our previous data obtained (Salvati MV 1999). Similar to our findings, Westerholm-Ormio M et al showed by *in situ* hybridation the higher density of IL-2 in lamina propria of family members of patients with CD and dermatitis herpetiformis (Westerholm-Ormio M 2002).

Then, we studied transcription factors involved in Th1 differentiation, such as T-bet. It is a crucial transcription factor involved in the early phase of Th1 differentiation. T-bet is able to control induction of IFNγ and repression of IL-4 and IL-5. Thus, T-bet initiates Th1 lineage development from naive Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs (Szabo SJ 2000). For T-bet RNA expression we found a significant increase in untreated coeliac mucosa if compared to control mucosa, similar results we showed for IRF-1, confirming our previous data (Monteleone I 2004). In potential CD, the

levels of T-bet were higher than controls but not statistically significant. Likely, the differentiation in Th1 starts in a early phase of disease, as showed in a model of uveitis in rat, in which T-bet were observed at 8 h, significantly increased 24 h, and decreased 48 h after lipopolysaccharide (LPS) injection (Li B 2006).

Moreover, we showed in potential CD mucosa a significant increase of IFNγ RNA expression. In particular, IFNγ levels correlated with intraepithelial lymphocyte infiltration, it agrees with previous data that showed higher density of IFNγ positive cells in potential CD patients with high γδ TCR+ intraepithelial lymphocytes (IELs) (Westerholm-Ormio M 2002).

Our data suggest that in potential CD despite the absence of mucosal damage T cells seem to be activated and differentiating towards a Th1 pattern, as supported by a significant increase of IL2, T-bet and IFNγ. We hyphotesize that regulatory mechanisms might contribute to prevent the progression towards a complete mucosal damage.

Therefore, we investigated the presence of immunoregulatory cytokines able to contrast the ongoing Th1 response in potential CD. We analysed to this purpose IL-10 expression, a key cytokine involved in immune regulation. The levels of IL-10 were significantly increased in T0 Marsh group of patients than controls, while in T1 Marsh group they were

reduced. In particular the ratio between IL 10 and IFN-γ was increased in group T0 if compared to active coeliac patients and group T1.

Further, we hypothesized that a crucial role in the maintainment of mucosal inflammation in untreated coeliac disease is determined synergistically by two immunological mechanisms: first, an excessive IFNγ production due to IRF-1/STAT-1 positive feed-back regulation and second, an inefficient immune suppression as evidenced by a low ratio IL-10/IFNγ.

Interestingly, in potential coeliac mucosa IRF-1 was significantly lower if compare to untreated CD. IRF-1 can have a protective role in intestinal inflammation, with a possible anti-inflammatory and/or restorative role. In fact a recent study shows that the administration of dextran sulphate sodium or trinitrobenzene sulfonic acid leads to a dramatic increase in lethality and colitis severity in IRF-1 knockout mice (Siegmund B 2004).

In conclusion in potential CD mucosa, T cells are committed and activated in the early phase of the disease (T0 Marsh). In this stage the presence of IL 10 producing lymphocytes down regulates IFNγ production. Subsequently, during the infiltrative phase (T1 Marsh), the down regulation of IL10 favours the up regulation of IFNγ production and thus might drive the mucosal damage.

A further data that support the hypothesis that the regulatory mechanisms might downregulate T cell mediate immuno-response in the early phase of CD enterophaty is the increased number of CD4+IL10+ cells

in mucosa of potential CD with T0 Marsh lesion. But in untreated CD the percentage of CD4+IL10+ cells was significantly down-regulated if compared to control, Marsh 1 and Marsh 0 patients. Very likely, IL-10 producing cells in untreated CD express CD8+ as suggested by Hammarstrom ML and colleagues (Forsberg G 2007).

Interestingly, a significant increase was observed in untreated CD and potential CD for CD4+CD25+FOXP3+ cells. Since the percentage of double positive CD4+IL10+ cells is significantly higher in Marsh 0 potential CD if compared to control and untreated CD patients, very likely, the CD4+CD25+FoxP3+ population in potential CD is able to produce IL-10 and regulate Th1 immune response.

Our data suggest that regulatory mechanisms play a crucial role to downregulate the inflammation in early phase of CD, the subsequent events leading to failure of these regulatory mechanisms remain to know.

IL2 RNA expression in duodenal mucosa

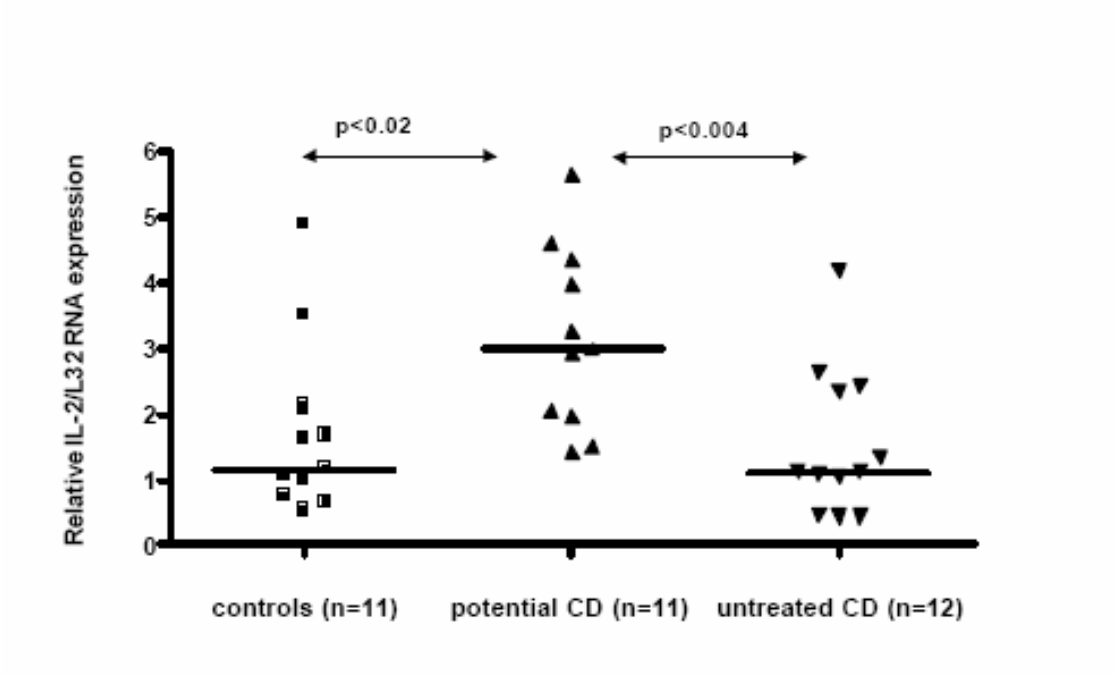


Fig. 1 IL2 RNA expression is significantly increased in duodenal mucosa from potential CD patients if compared to control ($p<0.02$) and to untreated CD patients ($p<0.004$). There are not differences between untreated CD and controls. Each point represents the value (relative IL2/L32 RNA expression) of IL2 taken from a single subject. Horizontal bars indicate median values.

T-bet RNA expression in duodenal mucosa

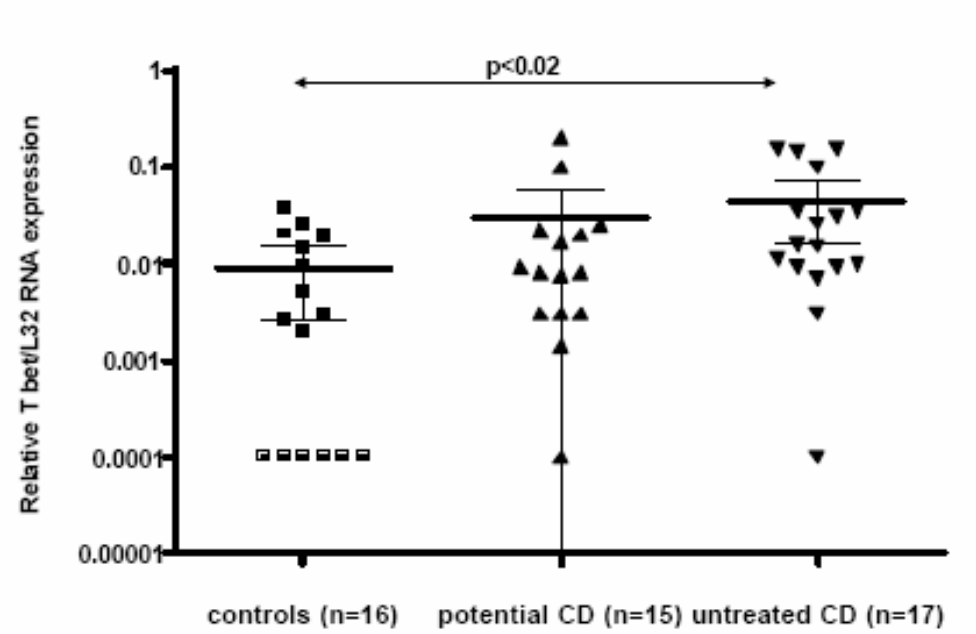


Fig. 2 T-bet RNA expression is significantly increased in duodenal mucosa from untreated CD patients if compared to controls ($p<0.02$). In potential CD patients there is a wide confidence interval, for this reason means are not different neither compared to controls nor to untreated CD patients. Each point represents the value (relative T-bet/L32 RNA expression) of T-bet taken from a single subject. Horizontal bars indicate median values.

IRF-1 RNA expression in duodenal mucosa

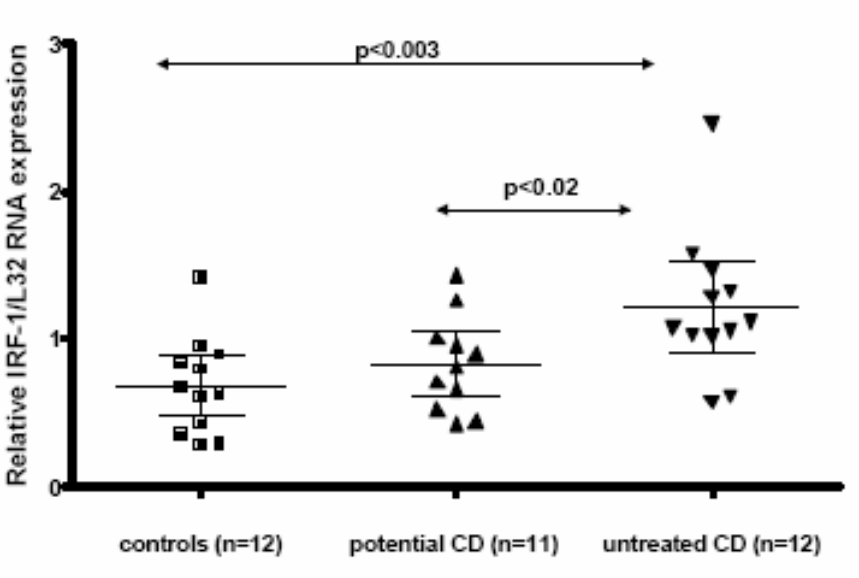


Fig. 3 IRF-1 RNA expression is significantly increased in duodenal mucosa from untreated CD patients if compared to controls ($p<0.003$) and to potential CD patients ($P<0.02$). Each point represents the value (relative IRF-1/L32 RNA expression) of IRF-1 taken from a single subject. Horizontal bars indicate median values.

IFNγ RNA expression in duodenal mucosa

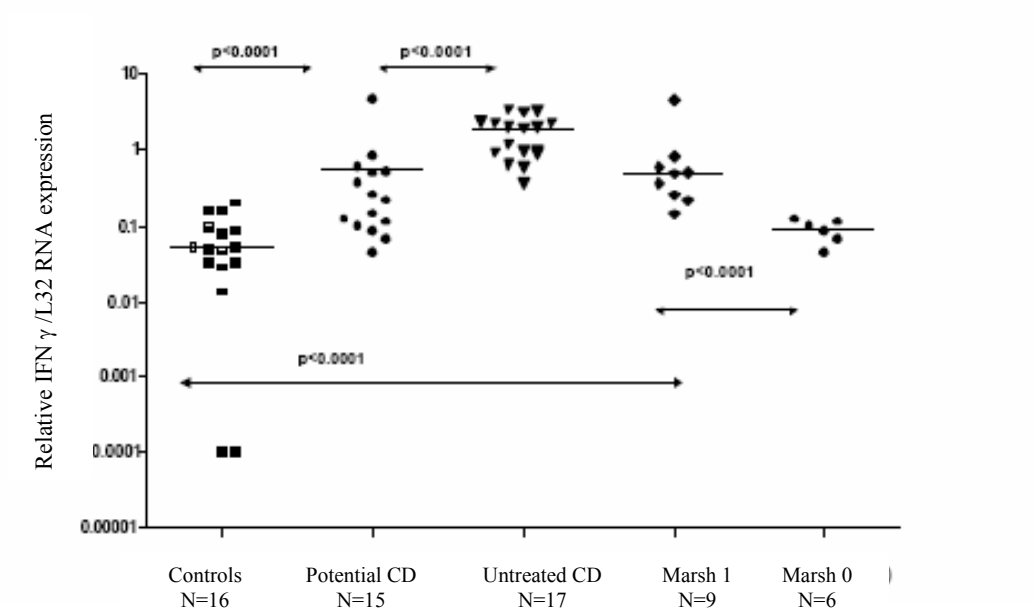


Fig. 4 IFNγ RNA expression is increased in duodenal mucosa from untreated CD patients if compared to controls and potential CD (0.00001 and 0.0001 respectively).
In potential CD patients with Marsh 1 intestinal lesion (Marsh 1) there is a significant increase of IFNγ RNA expression if compared to controls and potential CD with Marsh 0 (Marsh 0) ($p<0.0001$). Each point represents the value (relative IFNγ/L32 RNA expression) of IFNγ taken from a single subject. Horizontal bars indicate median values.

Correlation between IFNγ expression and intraepithelial lymphocyte infiltration in duodenal mucosa of potential CD patients

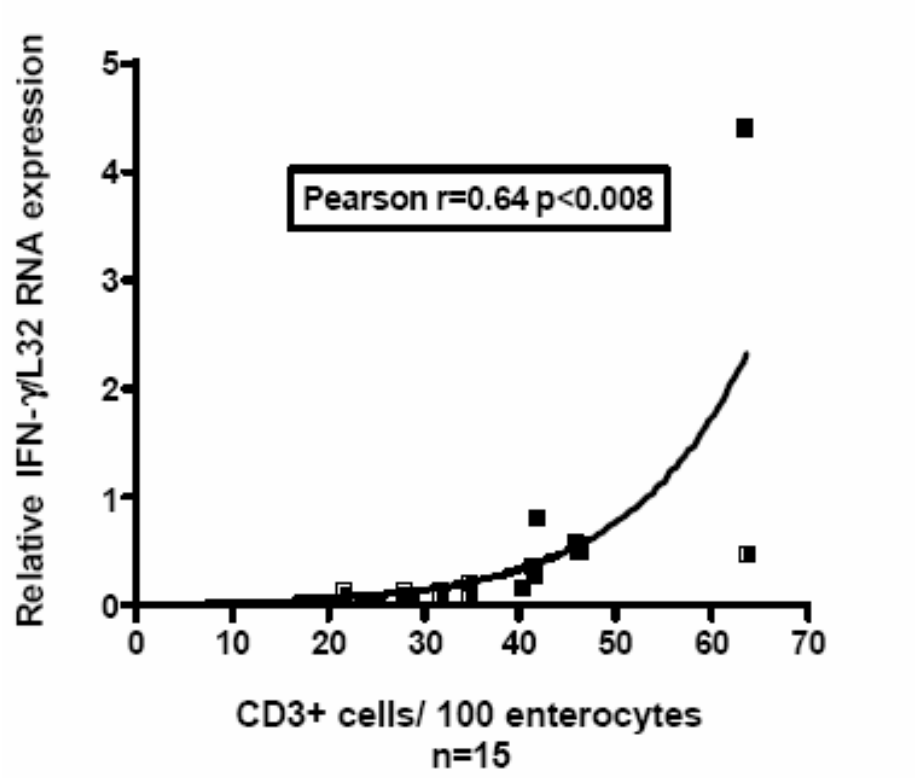


Fig. 5 IFNγ expression in potential CD mucosa correlates with intraepithelial lymphocyte infiltration ($p<0.008$, Pearson $r=0.64$)

IL-10 RNA expression in duodenal mucosa

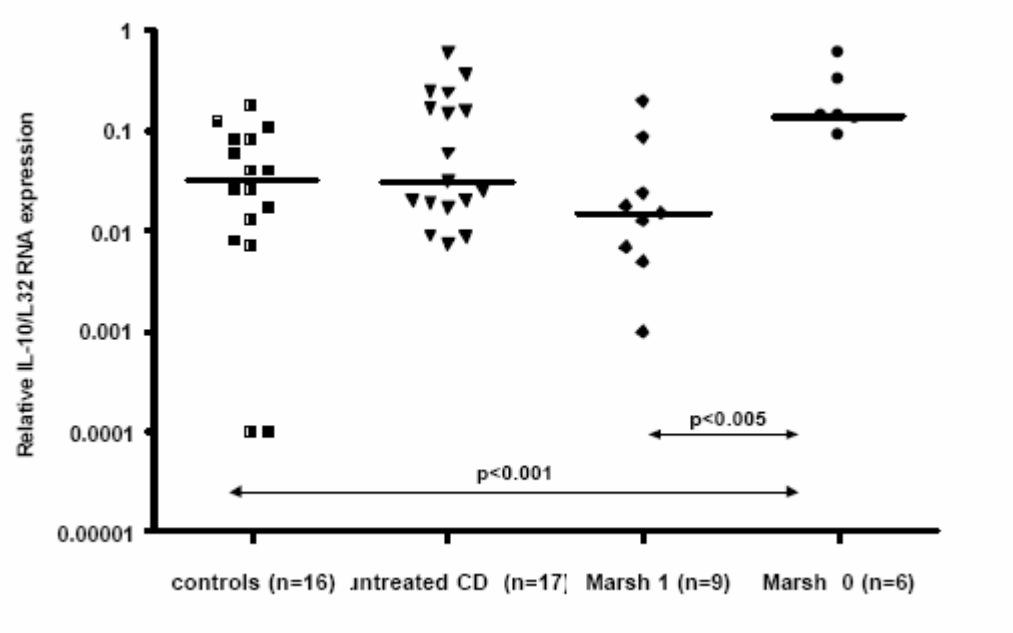


Fig. 6 IL10 RNA expression is increased in duodenal mucosa from potential CD patients with Marsh 0 lesion if compared to Marsh 1 lesion and controls (0.005 and 0.001 respectively).

IL-10 / IFN-γ Ratio in duodenal mucosa

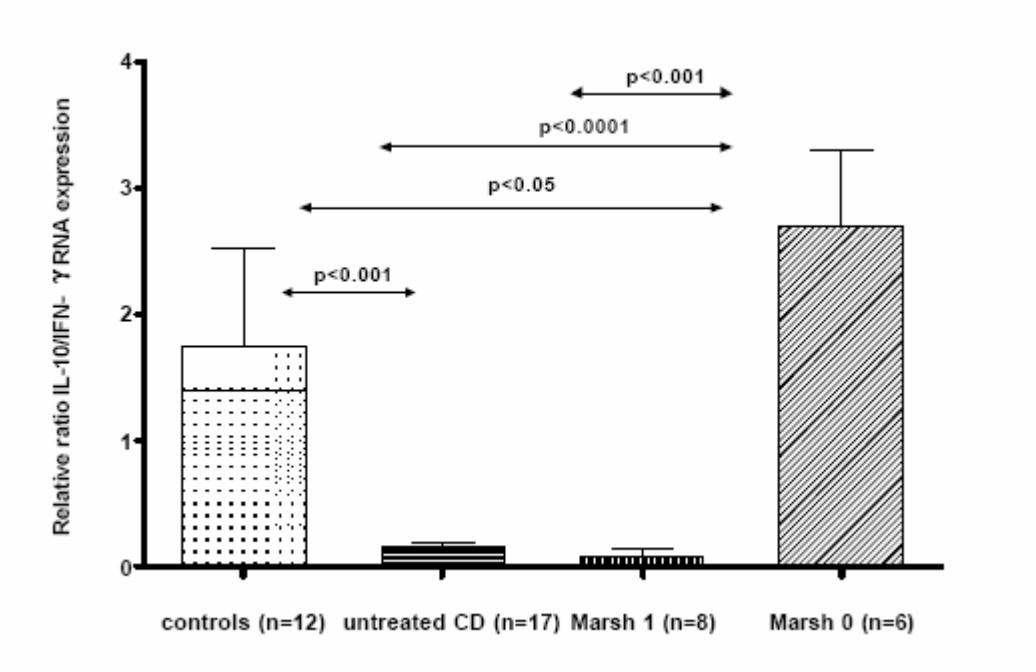


Fig. 7 IL-10 / IFN-γ ratio is higher in Marsh 0 potential CD than Marsh 1 potential CD, untreated CD and controls ($p<0.001$, $p<0.0001$ and $p=0.053$ respectively).

Phenotype of IL 10 producing cells in duodenal mucosa

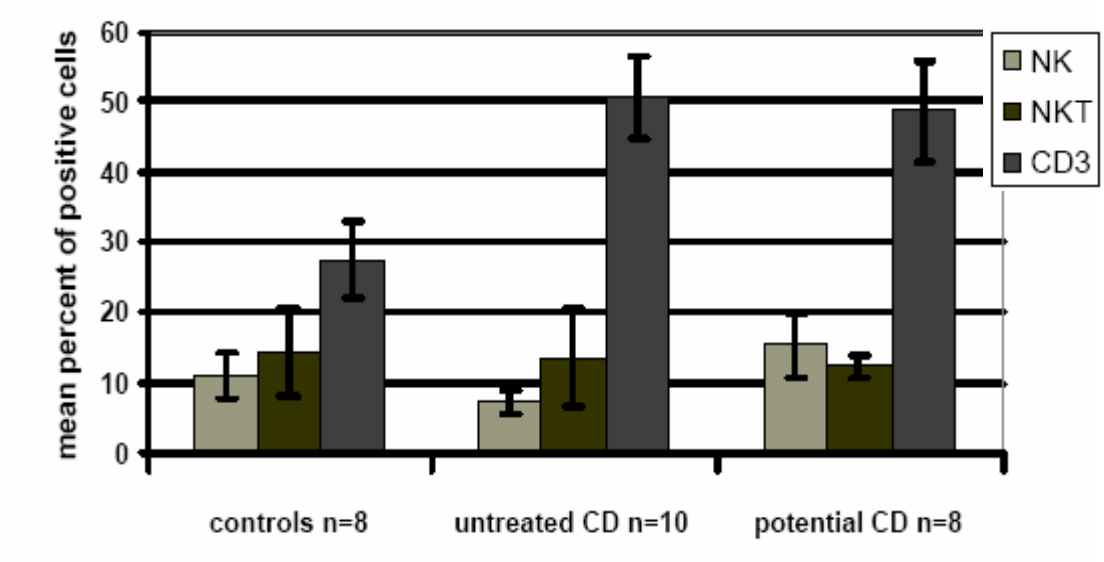


Fig. 8 We analysed in the gate of IL10+ cells the percentage of CD3+CD56- (T cells), CD3-CD56+ (NK) and CD3+CD56+ (NKT cells). The mean percentage of IL10 positive cells is significantly higher in the CD3+ CD56- population.

CD4+IL10+ T cells in duodenal mucosa

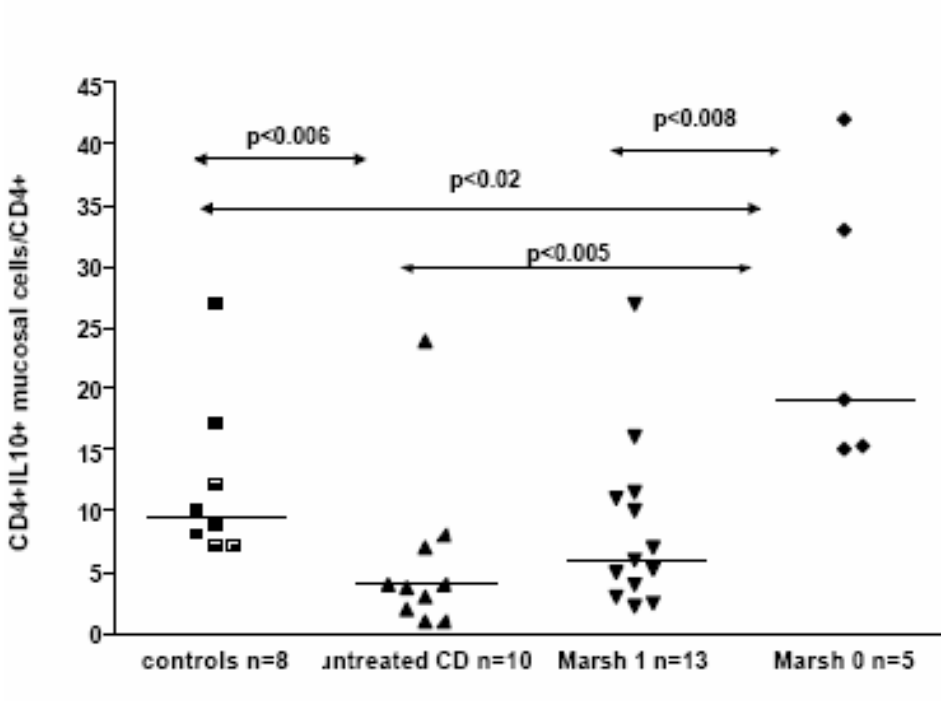


Fig. 9 Increased percentage of CD4+IL10+ T cells in duodenal mucosa from potential CD patients with Marsh 0 lesion compared to controls, untreated CD and Marsh 1 patients ($p<0.03$, $p<0.001$, $p=0.07$ respectively).
In untreated CD patients CD4+IL10+ cells are down-regulated than controls, Marsh 1 and Marsh 0 patients ($p<0.004$, $p<0.04$, $p<0.001$ respectivaly).

CD4+IL10+IL4+ (Th2) cells in duodenal mucosa

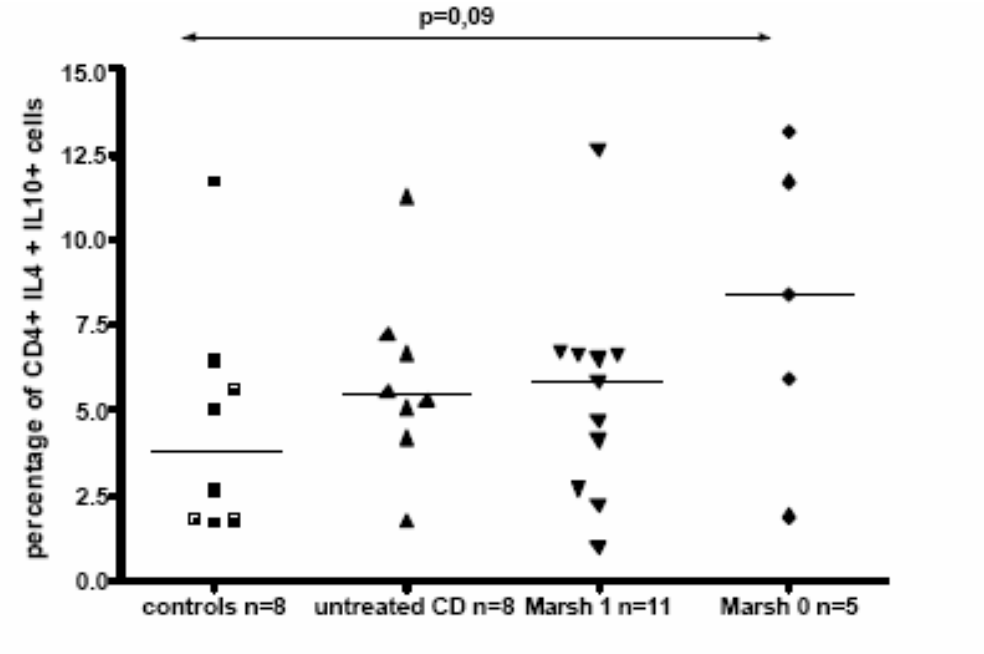


Fig. 10 The percentage of CD4+IL10+IL4+ cells is not significantly different in all groups analysed.

CD4+IL10+IL4- (Tr1) cells in duodenal mucosa

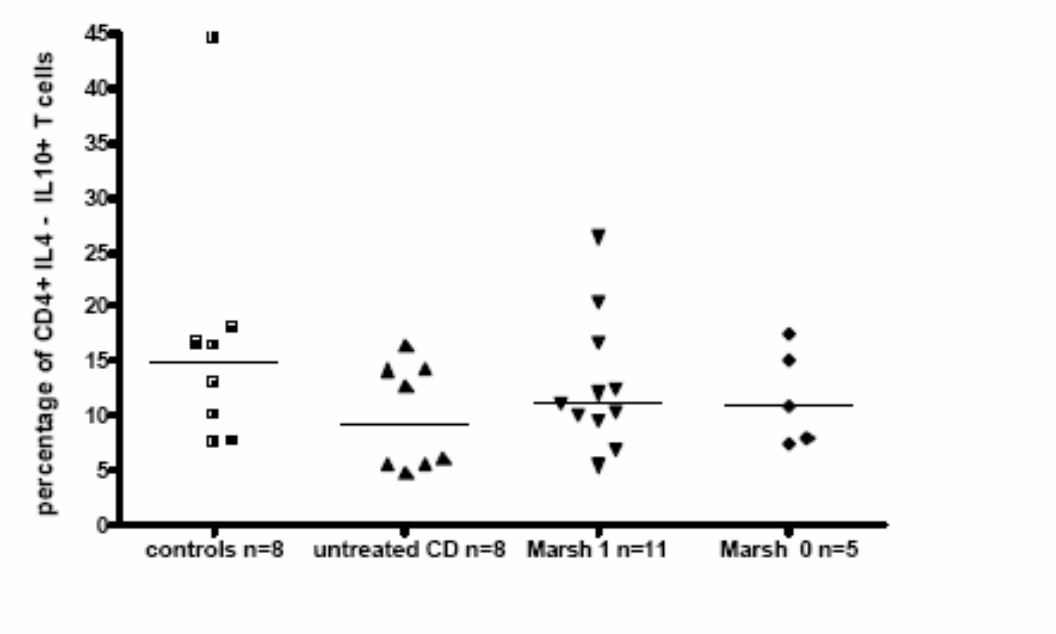


Fig. 11 The percentage of CD4+IL10+IL4- (Tr1) cells is not significantly different in all groups analysed.

CD4+CD25+Foxp3+ cells in duodenal mucosa

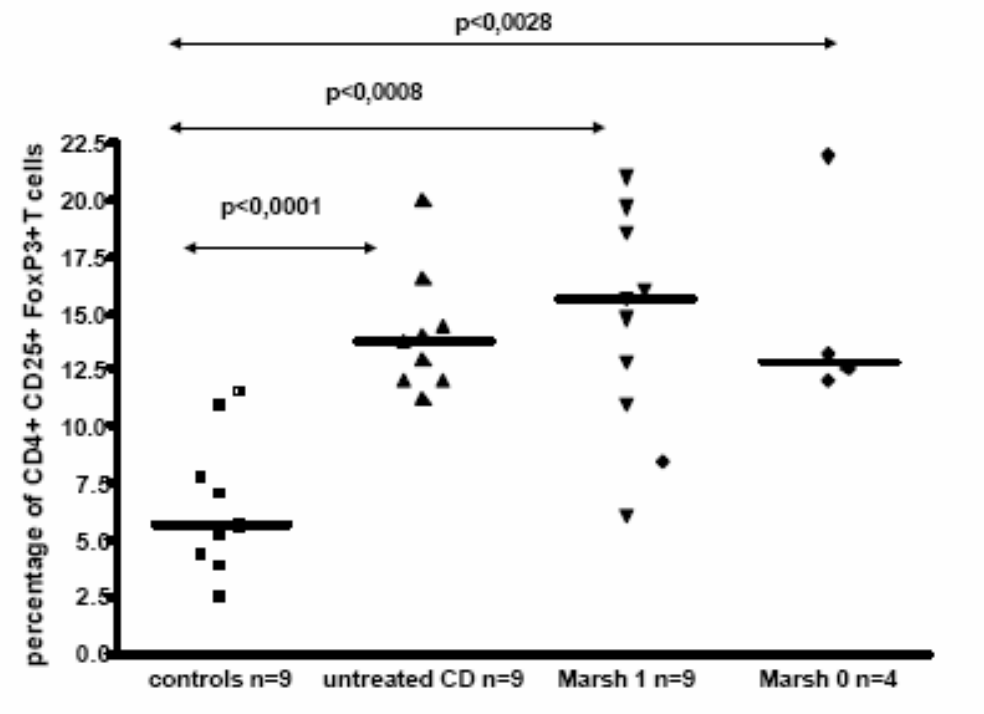


Fig. 12 Increased percentage of CD4+CD25+Foxp3+ cells in duodenal mucosa of untreated CD, Marsh 0 and Marsh 1 potential CD compared to controls ($p<0.0001$, $p<0.005$ and $p<0.001$ respectively).

4. IMMUNOLOGIC MARKERS IN COELIAC PATIENTS WITH SELECTIVE IGA DEFICIENCY.

Finally, we focused our interest on a particular condition, the CD in patients with selective IgA deficient (IgAD). Screening for CD is a special challenge in IgAD since the IgA class CD associated autoantibodies are not produced. In addition, enhanced T cell activation is present in the intestine of IgAD making difficult the differential diagnosis with potential CD. This study had the aim to find immunological markers that could improve the diagnostic accuracy of CD in IgAD subjects.

Introduction

Selective IgA deficiency (IgAD) is the most common primary immunodeficiency, characterized by total IgA serum level below 0.05 g/L with IgM and IgG serum remaining within normal ranges and absence of IgA in the secretions of the patients (Klemola T 1987). It is a heritable condition associated with the ancestral haplotype HLA-A1, Cw7, B8, DR3, DQ2, occurring with a prevalence of 1:300-1:800 in the European normal population (Koistinen J 1975, Vorechovsky V 2000). This condition may predispose to recurring respiratory, urinary, or gastrointestinal tract infections, as well as allergy and autoimmune disorders, although

frequently subjects with IgAD are asymptomatic (Cunningham-Rundles C 2001).

Secretion of IgA is the most important defence mechanism of the mucosal surfaces against microbial infections and penetration of foreign proteins. However, in IgAD the lack of IgA containing cells is compensated by an excess of cells containing IgM in the small intestinal and rectal mucosa (Savilahti E 1973). The efficacy of IgM on mucosal surfaces is not well established. IgA deficient individuals often have high levels of antibodies to cow's milk proteins and to other food proteins and increased levels of immunocomplexes containing milk proteins in their sera (Cunningham-Rundles C 1981). After oral poliovirus vaccination the patients with IgAD have an impaired capacity to eliminate poliovirus (Savilahti E 1988). In addition, enhanced T cell activation is present in the intestine of these subjects; in fact, they have a significant increase of CD25+ cells and high density of intraepithelial lymphocytes (IELs) in the jejunal mucosa (Klemola T 1988, Klemola T 1995). Finally, some of them have minimal morphological changes in the duodenal mucosa, which are recognizable only at the ultrastructural level (Giorgi PL 1986).

IgAD is frequently associated with coeliac disease (CD) and a 10- to 20-fold increased risk for CD may be observed in subjects affected by IgAD. Moreover, patients with IgAD have a higher incidence of silent forms of CD (Cataldo F 1998). For this reason, screening for CD is mandatory in

patients with IgAD, but it represents a special challenge since the specific IgA-class autoantibodies against gliadin (AGA), endomysium (EMA), and tissue transglutaminase (TG2) are not detectable. Determination of the IgG class of CD associated antibodies has been suggested as an alternative for identification of IgA deficient patients with CD, but the accuracies of these assays vary. IgG-TG2 measurement by enzyme-linked immunoassorbent assay using recombinant human (rh)-TG2 as antigen have proved to be highly reliable serological tests for the diagnosis of CD in patients with IgAD (Korponay-Szabo IR 2003), despite the diverse commercial methods present significant differences in terms of sensitivity and specificy (Villalta 2007).

More recent observations that histology has lost specificity for the diagnosis of CD have further increased the diagnostic difficulties; from a histological point of view, in fact, it is now clear that the development of the CD mucosal lesion is a dynamic process that may present in various stages or types of lesions (Marsh MN 1992). Thus, a minor abnormalities of jejunal mucosa, that frequently are presented in subjects with IgAD could reflect early and incomplete manifestations of coeliac disease. Several markers of gluten sensitivity have been proposed, such as the increased densities of IEL γδ+ or the count of IEL at the villous tip, but so far no single marker is considered a reliable indicator of this condition (Maki M 1991, Jarvinen TT 2004). More recently it has been shown that intestinal deposits of anti-TG2

would have the best sensitivity and specificity to predict the evolution to over CD (Salmi TT 2006).

In this study we investigated immunohistochemically jejunal biopsy specimens from patients with IgAD with or without associated CD with the aim to find markers that could improve the diagnostic accuracy of CD in IgAD subjects. In particular the density of intraepithelial γδ T cells and the deposition of anti TG2 IgM antibodies in duodenal mucosa were studied.

Patients

In this study jejunal biopsy specimens from 23 patients with IgAD (11 boys and 12 girls, mean age 7.2 years; range: 1.2 – 23.3 years) were investigated. Immunoglobulins were measured in serum of all patients, and in those with serum IgA concentration below 0.05 g/l in the presence of normal levels of serum IgG and IgM (>7 g/l and > 0.5 g/L, respectively) the diagnosis of IgAD was confirmed. None of the patients received drugs like phenytoin, penicillamine or captopril that might have affected the immunoglobulin levels. All subjects gave informed consent to the proposed study. Biopsy specimens from duodenum were obtained by upper gastrointestinal endoscopies or by Watson biopsy device. Seven of these patients (mean age: 7.6 years; range: 1.2 – 16 years) showed normal villous architecture in small intestinal mucosa (Marsh 0-1) (Oberhuber G 1999**)** and none of these had increased serum anti TG2 IgG antibodies on gluten containing diet. All

patients had clinical features suggestive of CD, such as recurrent non-specific diarrhoea (n=3), abdominal pain (n=2), iron-deficiency anemia (n=2), weight loss (n=1), anorexia (n=1). Seven patients (mean age: 6.8 years; range: 1.5 – 16.4 years) with IgAD were characterised by the presence of anti TG2 IgG antibodies in the serum and a normal architecture of the small intestinal mucosa (Marsh 0-1) on gluten containing diet. HLA class DRB1 and DQB1 molecules were determined in 5 patients and 5/5 carried the CD-associated haplotypes (DQ2)**.** They were considered compatible with diagnosis of potential CD. Four of these patients were first-degree relatives of coeliac disease. In five patients there were clinical features suggestive of CD: two patients presented intermittent diarrhoea, two iron-deficiency anemia and two elevation of serum aminotransferases and one failure to thrive. Nine IgA deficient patients (mean age: 7 years; range: 1.2 – 23.3 years) with subtotal or severe partial atrophy of duodenal mucosa and increased serum anti TG2 IgG antibodies were considered compatible with diagnosis of untreated CD. 3/3 were positive for HLA DQ2.

The study control group included immunologically intact patients with normal serum levels of immunoglobulins. From sixteen control patients (5 girls and 11 boys; mean age: 5.1 years; range: 1 – 11.2 years) with normal intestine and absence of serum IgA EMA and/or anti-TG2 autoantibodies jejunal biopsy specimens were studied by immunohistochemical analysis

for the density of CD25+ cells in the lamina propria, CD3 + and γδ+ intraepithelial lymphocytes in the epithelium and the expression of ICAM-1 and crypt HLA-DR. Control patients underwent esofagogastroduodenoscopy because of the presence of upper gastrointestinal symptoms (general diagnosis were: gastroesophagel reflux, chronic gastritis and gastric polyposis).

Anti-TG2 IgM intestinal deposits were investigated in jejunal mucosa of a subgroup of eight subjects of controls described above, twelve subjects (7 girls and 5 boys; mean age: 6.11 years; range: 2 – 12 years) with CD in accord to ESPGHAN criteria (Working Group of ESPGAN. 1990) and of nine potential CD (4 girls and 5 boys; mean age: 8.8 years; range: 3 – 16 years), characterised by the presence of coeliac specific autoantibodies (EMA and/or anti TG2) of IgA class, the presence of HLA DQ2 and/or DQ8, but a histologically normal mucosa.

Results

The densities of CD25+ cells in the lamina propria and the expression of ICAM-1 and crypt HLA-DR of jejunal specimens

The density of lamina propria CD 25+ cells were determined in 21 patients with IgAD: 8 subjects with absence of anti TG2 IgG antibodies and a histologically normal jejunal mucosa (IgAD), 7 potential CD with

presence of serum TG2 IgG and a histologically normal jejunal mucosa (Pot.CD-IgAD), 6 CD subjects with subtotal or severe partial atrophy of duodenal mucosa and increased serum anti TG2 IgG antibodies (CD-IgAD). In 17 out of 21 (83%) specimens from IgAD patients (7/8 IgAD, 4/7 Pot.CD-IgAD and 6/6 CD-IgAD) the number of lamina propria CD25+ cells was increased if compared to normal values (< 4 cells / mm2 lamina propria).

In IgAD without associated CD the density of lamina propria CD25+ cells (mean 15.15, median 11) was significantly higher ($p < 0.05$) than control patients (mean 6, median 4.5), but significantly lower than IgAD patients with CD (mean 111, median 110; $p< 0.005$). On the contrary, similar densities of CD25+ cells were present in the lamina propria of jejunal biopsy specimens from IgAD without associated CD and IgAD patients with potential CD (mean 16, median 6; $p=0.5$). Also CD IgAD patients has more CD25+ cells in the lamina propria compared to controls ($p<0.01$), while there were not differences significantly between potential CD IgAD patients and controls ($p=0.4$). (Fig.1)

The expression of lamina propria ICAM-1 was enhanced in 5 of 6 (83%) of IgAD patients without associated CD, in 4 of 4 (100%) of IgAD patients with CD, and in 2 of 6 of IgAD patients with potential CD (33%). The difference of expression was statistically significant between samples from IgAD without associated CD patients and controls ($p < 0.05$). Instead,

the expression did not differ significantly between IgAD patients without CD and IgAD patients with potential CD and CD.

Positive staining of crypt epithelial cells with anti-HLA DR was similar in IgAD patients without CD (6/6), potential CD (4/5) and CD subjects with IgAD (4/4). The expression of HLA-DR positive cells did not differ in these three groups and controls.

Infiltration of lymphocytes in the epithelial compartment of jejunal specimens

CD3 + intraepithelial lymphocytes (IELs)

The number of CD3+ lymphocytes was determined in 19 patients with IgAD: 8 IgAD without CD, 7 potential CD, 4 CD subjects. The density of CD3+ cells in the epithelial compartment was higher than cutoff (> 34 cells/ mm epithelium) in all IgAD CD (4/4), but only in 2/7 (28%) potential CD and in 5/8 (62%) IgAD patients without CD.

The IgA deficient patients without CD had more CD3+ cells in the epithelium (mean 36.7, median 40.15) compared to control patients (mean 22.31, median 21.15; $p< 0.05$), but significantly the density was lower than IgAD patients with CD (mean 82.12, median 86.5; $p< 0.01$). Interestingly, the density of CD3+ intraepithelial lymphocytes did not present difference statistically significant between IgAD without CD and potential CD subjects (mean 32, median 29; $p= 0.7$). CD IgAD patients has more CD3+

cells in the epithelium compared to controls ($p<0.005$), while in potential CD IgAD also if the number of CD3+ cells tended to be higher, there were not differences significantly compared to controls ($p=0.07$). (Fig. 2).

T cell receptor (TCR) γδ intraepithelial lymphocytes

The number of TCR γδ IELs was detected in jejunal specimens of 20 patients with IgAD: 8 without associated CD, 7 potential CD, 5 CD subjects. All IgAD CD patients had an increase of TCR γδ IELs in the jejunal epithelium (5/5), but only 3/7 (42%) potential CD and 4/8 (50%) patients without CD if compared to normal values (> 3.6 cells / mm epithelium).

In IgAD without associated CD (mean 6.37, median 4) the number of TCR γδ IELs was significantly raised compared with control patients (mean 1.6, median 1.25; $p< 0.05$), but significantly lower than CD IgAD patients (mean 24.74, median 24.8; $p< 0.005$). The density of TCR γδ IELs was similar in IgAD and potential CD IgAD (mean 4.38, median 2.7; $p=0.5$). In CD IgAD patients the density of γδ IELs was higher than to controls ($p<0.005$), while there were not differences significantly between potential CD IgAD patients and controls ($p=0.05$), also if in the potential CD IgAD patients the number tended to be increased.

Finally, in IgAD without CD (mean 16.7, median 17.1) the γδ+/CD3+ ratio was significantly higher ($p<0.05$) than in controls (mean 8.9, median 8.4), while it was not different between these patients and subjiects with

potential CD and CD (mean 14.7, 27.7; median 9.2, 29.7; p=0.7, p=0.1 respectively). In the CD patients the γδ+/CD3 ratio was significantly increased ($p<0.05$) than in controls, but there were not differences significantly in the potential CD compared to controls (p=0.2) (Fig.4).

Anti TG2 IgM intestinal deposits in patients with IgAD

In 19 patients with IgAD anti TG2 IgM deposits were investigated. Jejunal mucosa from non coeliac subjects (5/5) showed IgM only inside plasma cells and epithelial cells. On the contrary, 6/7 untreated coeliac patients showed IgM deposits below the villous and crypt basement membranes, corresponding to the intestinal localization of TG2, apart from IgM inside plasma cells. In these specimens we observed a patchy distribution and less thick bands along the surface and crypt basement membranes and around mucosal vessels of the deposits. Only one patient presented increased density of these bands. Among the group of potential coeliac patients, anti-TG2 IgM deposits were noted only in one of 7 patients. In this patient the pattern was similar to that described for patients with IgAD and CD. In CD patients the prevalence of intestinal anti-TG2 deposits was significally higher than potential CD and patients without CD ($p< 0.01$, $p< 0.005$ respectively) (Tab. 1).

All positive samples showed a patchy IgM deposits pattern, were analysed by confocal microscope to further asses the colocalization of IgM

deposits with TG2. Confocal analysis confirmed in all cases the observations previously described.

In the control group of immnologically intact patients, coeliac subjects on gluten-containing diet showed elevated levels of IgM containing cells in the jejunal mucosa but anti TG2 IgM intestinal deposits were seen only in 6/12 and in 1/9 potential CD patients. In all these positive specimens for intestinal deposition of antiTG2 IgM we observed a patchy distribution and less thick subephielial bands None of controls (8/8) presented deposits.

Discussion

Findings of enhanced T cell activation are present in the intestine of IgAD patients. These subjects often shows pathological changes in the small intestinal mucosa, some of which are recognizable only at the ultrastructural level (Giorgi PL 1986). In addition, the density of intraepithelial lymphocytes and of activated CD25+ cells in both the lamina propria and in the surface epithelium are increased in the jejunal mucosa of patients with IgAD (Klemola T 1988, Klemola T 1995). Our results confirmed these previous data; in fact, we found an increase of the number of lamina propria CD25+ cells and the expression of ICAM-1 and crypt HLA-DR in IgAD independently from the diagnosis. In particular, IgAD patients without associated CD showed a density of lamina propria CD25+ cells higher than immunologically intact controls. The pathogenesis of these

mucosal lesions is not well understood. In IgAD the secretory IgA is severely diminished or lacking in their external secretions. Secretion of IgA is the most important defence mechanism of the mucosal surface against virus, bacteria and toxins. IgA also can complex with antigens that have penetrated the lamina propria and transport them across epithelial cells to facilitate antigen exclusion (Cunningham-Rundles C 2001). This lack of secretory IgAD is replaced by a compensatory increase in secretory IgM (Natvig IB 1997). Although secretory IgM has been shown to be functionally active, it is not clear that it confers the same mucosal protection as secretory IgA. IgA deficient blood donors harbour poliovirus in their intestine longer after oral vaccination, while they reached the same serum antibody levels than normal subjects, confirming the hypothesis it is due to an inefficiency of local mucosal immunoglobulins and not due to an inadequate serologic response (Savhilati E 1988). Over 60% of patients with IgAD have precipitating antibodies to bovine milk proteins and circulating immune complex, in which milk proteins are important antigenic elements, probably owing to increased penetration of food proteins through the intestine (Cunnigham-rundles C). It is demonstrated an abnormal intestinal permeability in these subjects (Pignata C 1990). Finally, IgA in the serum does not fix complement by classical pathway, although it can do so as a aggregate by the alternative pathway, it has been suggested that IgA acts as a “silent housekeeper”, in which foreign antigen are bound by IgA

into complexes and removed by the phagocytic system, but with little or no resultant inflammation (Jacob CM 2008). On the contrary, IgG antibodies are able efficiently to eliminate antigen by promoting phagocytosis, but they may maintain inflammatory and tissue-damaging processes by complement activation. IgA patients with a history of frequent respiratory tact infections respond to an oral cholera vaccination with significantly higher intestinal cholera toxin-specific IgG and IgM antibody responses than healthy IgAD individuals and controls (Friman V 1994). In conclusion, damage to the small intestinal mucosa in patients with IgAD could derive from abnormal microbiological or immunological processes.

Interestingly, we found increased number of intraepithelial lymphocytes, in particular of CD3+ and γδ+ cells in the epithelial compartment of IgA deficient patients without associated CD, but the density was lower than IgAD patients with CD, while it did not present difference statistically significant compared to potential CD IgA deficient subjects. In addition, our IgAD patients without CD showed a higher γδ+/CD3+ ratio than controls, while this ratio was similar to subjects with potential CD and CD. These data do not agree with results of previous studies; in these all IgAD patients with normal jejunum had a density of CD3+ and γδ+ cells similar to immunologically normal controls, only IgAD patients with a low frequency of infections showed an increase of γδ+ IELs, suggesting a compensatory mechanism of surface protection (Nilssen DE 1993). Instead,

Klemola T et al (Klemola T 1995) found an increased number of γδ+ lymphocytes only in the jejunum of patients with IgAD and CD during gluten free diet when their jejunal morphology was normal. All our IgA deficient patients with absence of serum IgG associated-CD antibodies and normal mucosa architecture at the intestinal biopsy examination had clinical features suggestive of CD, such as recurrent non-specific diarrhoea, abdominal pain, iron-deficiency anemia, weight loss, anorexia. Thus they could have an early CD.

It is earlier observed that an increased density of γδ+ IELs (Iltanen S 1999) or the count of IELs at the villous tip in the jejunal epithelium (Salmi TT 2006, Jarvinen TT 2004) predicts the forthcoming coeliac disease in autoantibody positive patients with a normal villous structure. In particular Paparo et al (Am J Gastroenterol 2005) suggested that a high γδ+/CD3+ ratio of intraepithelial lymphocytes would be a marker of genetic gluten intolerance. However a high count of γδ IELs is not specific for CD and also if the ratio γδ+/CD3+ indeed differentiates early developing CD from non-coeliac controls, there was a considerable overlapping between the groups (Spencer J 1991, Kaukinen K 2006).

Certainly, the detection of intestinal deposits of IgA antibodies against TG2 strengthens the probability of CD in borderline cases where the histology is difficult to interpret (Salmi TT 2006). It is well know that anti-TG2 IgA autoantibodies are produced in the intestinal mucosa (Marzari

2001) and they can deposit on extracellular TG2 in the small-bowel mucosa even when not measurable in serum (Picarelli A 1996). In the case of IgA deficiency, IgA deposits were missing (Korponay-Szabo IR 2003). IgAD is characterized by an excess of cells containing IgM in the small intestinal (Savilahti E 1973). Thus, we investigated the IgM antibodies against TG2 deposits in the small intestinal mucosa of patients with IgAD to find the best specific disease markers able to distinguish CD patients among IgAD subjects. We found that 86% (6/7) of CD IgAD patients presented specific intestinal deposits of IgM anti-TG2 antibodies, confirming previous data. In contrast with they, where the deposits were thick, with a clear localization below the basement membrane, along the villous and the crypt and around mucosal vessels in all patients with untreated CD (Korponay-Szabo IR 2004, Tosco A 2008), in our CD IgAD patients the positivity of intestinal IgM anti TG2 antibodies deposits was less clear, with tracts with evident deposits and tracts in which these deposits were absent. In the group of IgAD patients with potential CD only one subjects of seven showed these deposits with similar of distribution described for patients with IgAD and CD. This prevalence is not agreed with recent data in which 85% of patients with potential CD turn out to be positive for intestinal deposition of anti-TG2 IgA (Tosco A 2008). Also in our control group included patients with normal serum levels of immunoglobulins the data were in contrast with previous results. These differences of prevalence and distribution could

reside in a lower titer of IgM anti-TG2 antibodies and/or in a lower affinity of these antibodies for the intestinal transglutaminase.

Moreover, identification of gluten-sensitive individuals in the groups of patients with IgAD is necessary, because it cannot be ruled out that these individuals, if gluten sensitive, are at risk for secondary complications of untreated coeliac disease such as osteopenia and lymphoreticular malignancy. New methods are needed to make the diagnostic procedure easier.

The density of CD25+ cells in the lamina propria of duodenal mucosa

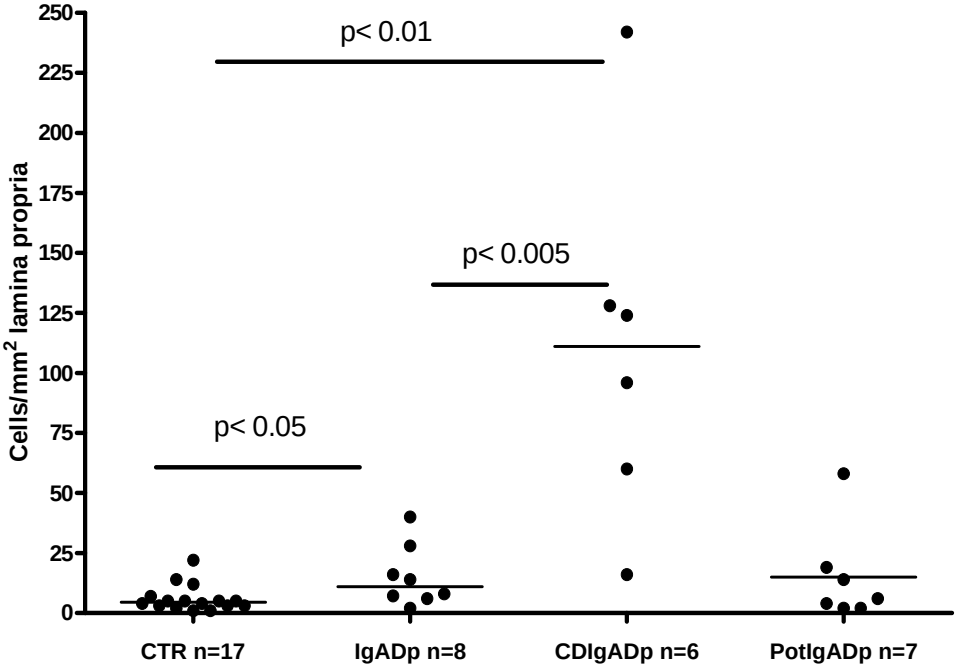


Fig. 1 Increased density of lamina propria CD25+ cells in IgAD without CD (IgADp) and in IgAD patients with CD (CDIgADp) if compared to controls ($p<0.05$ and $p<0.01$ respectively).
IgADp present lower density of lamina propria CD 25+ cells than CDIgAp ($p< 0.005$), but similar to potential CD (PotIgADp) ($p=0.5$). Each point represents the density (Cells/mm^2 lamina propria) of CD25+ cells taken from a single subject. Horizontal bars indicate mean values.

The number of CD3+ intraepithelial lymphocytes (IELs) in the epithelium of duodenal mucosa

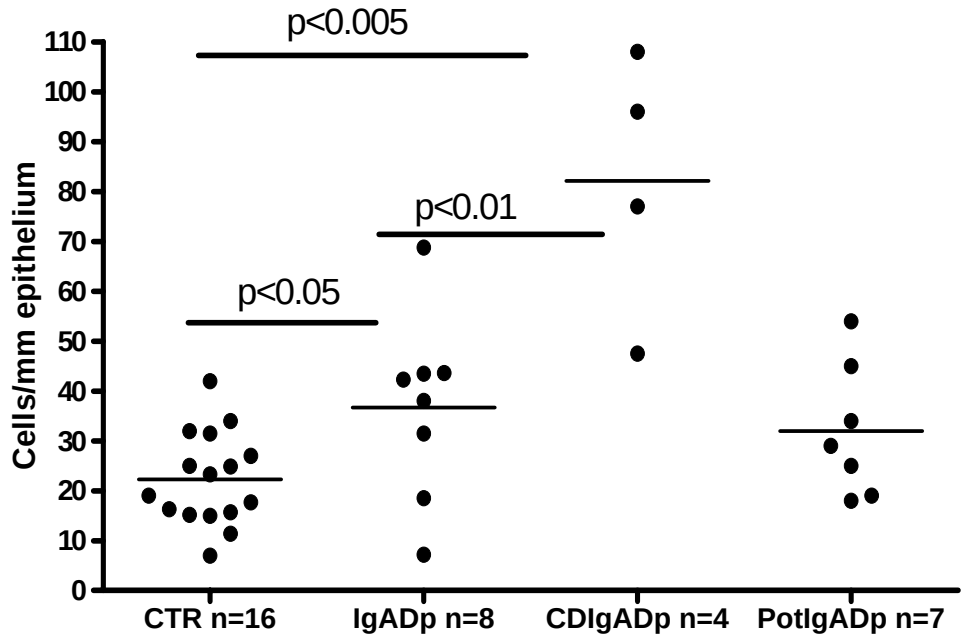


Fig. 2 Increased number CD3+ cells in the epithelium of IgAD without CD (IgADp) and IgAD patients with CD (CDIgADp) if compared to controls ($p<0.05$ and $p<0.005$ respectively).
IgADp present lower number of CD 3+ intraepithelial lymphocytes than CDIgAp ($p< 0.01$), but similar to potential CD (PotIgADp) ($p=0.7$). Each point represents the number (Cells/mm ephitelium) of CD3+ cells taken from a single subject. Horizontal bars indicate mean values.

The number of T cell receptor (TCR) γδ intraepithelial lymphocytes (IELs) in the epithelium of duodenal mucosa

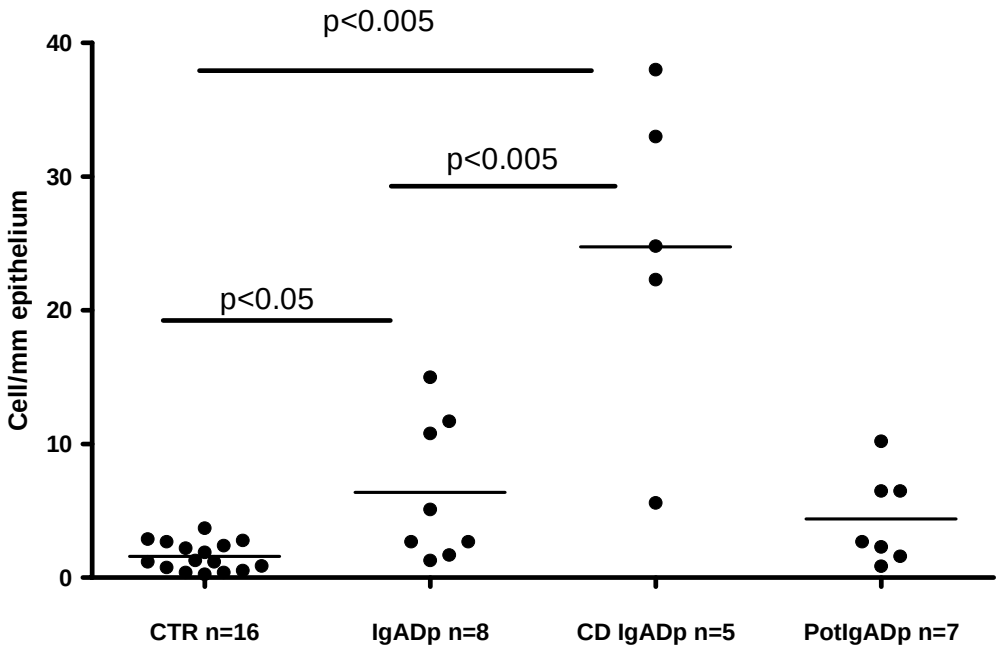


Fig. 3 Increased number γδ IELs in the epithelium of duodenal mucosa of IgAD without CD (IgADp) and IgAD patients with CD (CDIgADp) if compared to controls ($p<0.05$ and $p<0.005$ respectively).
IgADp present lower number of γδ IELs than CDIgAp ($p< 0.005$), but similar to potential CD (PotIgADp) ($p=0.05$). Each point represents the number (Cells/mm ephitelium) of γδ+ cells taken from a single subject. Horizontal bars indicate mean values.

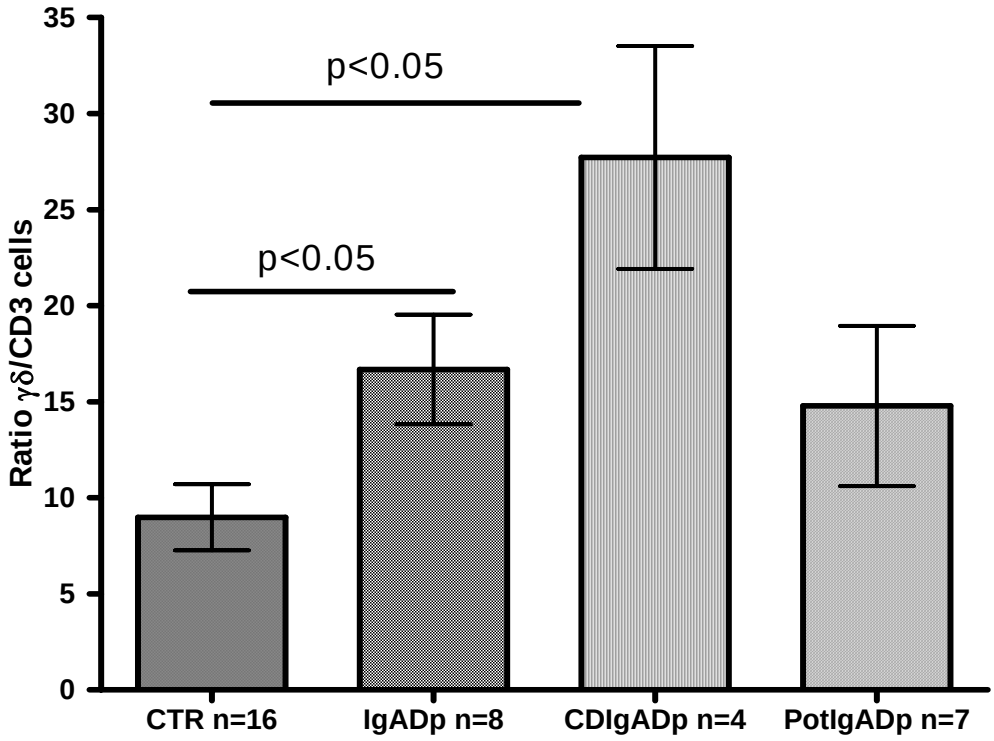


Fig. 4 In IgAD without CD the γδ+/CD3+ ratio was higher than in controls ($p<0.05$), while it was not different between these patients and subjects with potential CD and CD ($p=0.7$ and $p=0.1$ respectively). In the CD patients the γδ+/CD3 ratio was significantly increased ($p<0.05$) than in controls.

Tab. 1 Anti-TG2 IgG jejunal deposits in subjects with selective IgA deficiency

Patients	Prevalence positivity	% Positivity
IgAD without CD (IgADp)	0/5	0%
IgAD with serum TG2 and normal mucosa (potential CD)	1/7	14%
IgAD with CD *	6/7	86%

*** P < 0.005 vs IgADp and p < 0.01 vs potential CD**

METHODS

EMA AND ANTI-TG2 ANTIBODIES

Serum IgA and IgG (for patients with IgAD) EMA were detected by indirect immunofluorescence on 7-μm thick frozen sections of human umbilical cord. Sera were tested, diluted 1:5 and 1:50, and incubated for 30 min at room temperature. Serum was removed by washing with PBS solution, and the sections were exposed for 30 min to FITC-labeled rabbit anti-human IgA or IgG_1 (1:80 and 1:100 respectively). Samples were considered positive if a thin fluorescent network appeared around the smooth muscle fibres. Positive results were quantified further by titration.

Serum levels of IgA and IgG (for patients with IgAD) were determined by ELISA with a kit based on human recombinant antigen (Eu-tTg IgA and IgG; Eurospital, Trieste, Italy). Samples were diluited 1:26 before use and left to react in the wells for 60 min. Nonspecific antibodies were removed by washing. Horse radish peroxidase-labeled goat anti-human IgA or IgG were added to the wells and left to bind to the human antibodies; the excess conjugate was washed aways and a chromogenic substrate was added. After incubation for 20 min, we measured the optical density (450 nm) with a SpectraCount apparatus (Packarad Bioscience Company, USA).

HLA TYPING

Patients were genotyped for HLA class DRB1 and DQB1 molecules. A Dynal Allset^{+} SSP DR low resolution kit (Dynal Biotech Ltd, Wirral, UK), a Dynal Allset^{+} SSP DQ low resolution kit, a Dynal Allset^{+} SSP DQB103 and Dynal Allset^{+} SSP DQA1 were used for typing. Results were obtained after a 2% agarose gel electrophoresis.

DUODENAL BIOPSY AND IMMUNOHISTOCHEMICAL ANALYSIS

Biopsy specimens from duodenum were obtained by upper gastrointestinal endoscopies or by Watson biopsy device. At least three small intestinal biopsy specimens were obtained. Two fragments were fixed in 10% formalin, embedded in paraffin wax, sectioned at 5 μm thickness and stained with hematoxylin-eosin. The remaining fragment was immediately embedded in optimal cutting temperature compound (OCT-BioOptica), stored in liquid nitrogen. For the immunohistochemical study, biopsy specimens cryostat sections were cut at 4 μm and fixed in acetone for 10 min. After a 20 min pre-incubation with normal rabbit serum (1:100, Dako), sections were covered for 1 hr with anti-CD3 (1:200; Dako), anti-CD25 (1:20; Dako), anti-TCRγδ (1:80; Thema), anti HLA-DR (1:10; Dako) and anti-CD54 (ICAM-1) (1:200; Dako) monoclonal antibodies, followed by rabbit anti-mouse immunoglobulins for 30 min. Monoclonal

antibodies were diluted in Tris pH 7.4, all incubations were performed at room temperature in a humid chamber. As a negative control, primary antibody was replaced with mouse IgG2a/IgG1 (1:100; Dako). After washing with Tris pH 7.4, the sections were layered with monoclonal mouse PAP (peroxidase-antiperoxidase) (1:100; Dako) for 30 min. 2-amino-9-ethyl-carbazole (AEC) (Sigma) was used as peroxidase substrate. Finally, sections were counterstained with Mayer's hematoxylin and mounted with Aquamount (BDH, Poole, England).

The density of cells expressing CD3 and TCRγδ+ in the intraepithelial compartment was determined by counting the number of stained cells per mm epithelium. Cut-off values for CD3+ and TCRγδ+ cells are 35 mm/epithelium and 3,2 mm/epithelium respectively.

The number of cells expressing CD25 in the lamina propria was evalutated within a total area of 1 mm^2 of lamina propria. Cut-off value for CD25+ cells is 4 mm^2 lamina propria.

Staining of cells expressing HLA-DR and ICAM-1, in the crypt and the lamina propria respectivaly was graded as weak, moderate or strong. Slides were analysed by two observers who were blinded.

RNA EXTRACTION AND cDNA PREPARATION

RNA was extracted from biopsy specimens using 1 mL of a monophasic solution of phenol and guanidine isothiocyanate (Trizol, Invitrogen Italia

SRL) and chloroform, followed by isopropanol (Sigma-Aldrich SRL) precipitation. The integrity of RNA was checked by electrophoresis on a 1.5% agarose gel. A constant amount of total RNA (0.5-1 μg) was retro-transcribed into complementary cDNA in a 20 μl reaction mixture containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCL_2$, 500 μM each of dATP, dCTP, dTTP, and dGTP, 10 mM DTT, 0.5 μg oligo (dT) and 100 U MMLV-RT (Invitrogen Life Technologies). RNA was retro-transcribed into cDNA at 42° C for 60 min, the reaction was stopped at 70° C for 10 min and then the samples were cooled in ice.

ANALYSIS OF mRNA EXPRESSION BY QUANTITATIVE RT-PCR TAQ-MAN

Real time PCR was performed using TaqMan probes/primers developed by BioRad (Milan) on demand. Quantification was performed in duplicate wells using Biorad reagents (Supermix) for Icycler iQ Real Time PCR detection system (Biorad, Milan). L32 gene RNA was used as an endogenous control. As a calibrator sample we used human peripheral blood mononuclear cells stimulated with lypopolisaccharide (1mg/ml) in RPMI for 2 hours. In each experimental plate we analysed in the same experimental conditions the RNA marker and the house-keeping gene of both the patient and the calibrator sample. Real-time PCR analysis was conduced using 5μl di cDNA in a total reaction volume of 50μl

conteining: 1.5 U Platinum Taq DNA Polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP, 400 μM dUTP, 1 U UDG (Platinum Quantitative SuperMix-UDG, Invitrogen Life Technologies), 0.2 μM of each primer and 0.2 μM probe. Samples were initially warmed to 50°C for 2 min, then the Taq DNA polymerase was activated by heating to 95° C for 2 min. PCR amplification was performed using the following conditions: 35-45 cycles of denaturation for 15 s at 95°C, annealing 1 min at 58°C for IFN γ, L32 and at 57° C for IL-2, IRF-1, T-bet, L32). A calibration curve was created by two fold dilution with the calibrator sample for the RNA marker and for the L32 gene.

Once completed the amplification the iQ cycler program calculated the efficiency of the experiment. The quantification of the RNA marker was analysed by a comparative threshold cycle (C_T) method. ΔC_T stands for the difference between C_T of the marker gene and the house-keeping L32 gene. $\Delta\Delta C_T$ stands for the difference between C_T of the analysed sample and the C_T of the calibrator sample. Calculation of $2^{-\Delta\Delta CT}$ then gives the relative amount of the analysed sample compared to the calibrator both normalised to the endogenous control L32. This calculation was obtained by a Real Time quantification program kindly developed by BioRad.

ISOLATION OF LYMPHOCYTES FROM SMALL INTESTINAL BIOPSIES

Mucosal explants were either immediately incubated in a sterile tissue cultured dish (35 x 10 mm Falcon, Becton Dickinson, France) for a 1 h and 30 min period at 37°C in RPMI 1640 medium (Cambrex, Belgium), without serum, with 1mg/ml Collagenase-A (Roche). In order to promote intra-cellular cytokine retention, Brefeldin A (BFA) (10 ug/ml, Sigma-Aldrich) was added during the incubation.

INTRACELLULAR CYTOKINE DETECTION BY FLOW CYTOMETRY

Cells obtained from fresh biopsies were washed and labelled with conjugated mABs against surface antigens: an PE-Cy5-anti-CD3antibody(UCHT1), FITC-anti-CD56 antibody(NCAM16.2), Cy-anti CD4 antibody (RPA-T4) and anti-CD25 antibody (M-A251) all purchased from BD (Pharmingen, San Diego, CA, USA). Appropriate isotype-matched control antibodies(BD) were included in all experiments. After washing twice with PBS, cells were fixed and permeabilized with Cytofix/cytoperm (BD) according to the manufacturer's instructions, followed by staining with PE-anti IL10 antibody (JES3-9D7) (BD Biosciences) or FITC-anti Foxp3 antibody (eBioscience) or Alexa 488-anti-IL4antibody(8D4-8) (BD Biosciences) in permeabilization buffer and

then the cells were analyzed by a flow cytometer FACSCalibur using CellQuest Software (Becton Dickinson).

A first gate was performed on the basis of forward and side scatter profiles. Large granular cells were excluded in our gated population because of their very high side scatter.

INTESTINAL DEPOSITS OF ANTI-TG2 ANTIBODIES

Fixed in acetone, 5 µm frozen sections of each patient were examined by double immunofluorescence. After a 15 min preincubation with rabbit normal serum (1:100, Dako), the sections were covered with a monoclonal mouse antibody against guinea pig TG2 (CUB 7402) (1:200, NeoMarkers) for 1h at room temperature in a humid chamber. The sections were washed in PBS and incubated with a mixture of fluorescein isothiocyanate (FITC)-labelled rabbit antibody against human IgA and IgM (1:100, Dako), to detect (in green) IgA and IgM deposits, and R-phycoerythrin (RPE)-labelled rabbit anti-mouse antibody (1:40, Dako), to detect (in red) TG2, for 30 min in the dark. Finally, the section were washed several times in PBS and mounted by glycerol/PBS (1:10). The colocalization images of IgA and IgM mucosal deposits and TG2 that resulted in yellow were analysed by a confocal microscopy (LSM510, Zeiss).

DIETARY ASSESSMENT

The patients underwent a nutritional evaluation with the calculus of daily gluten intake amount and calcium, compared with a control population matched for age and sex. This evaluation was made with a “frequency questionnaire” and alimentary history. The amount of gluten introduced with aliments was calculated multiplying the total of vegetal protein for 0.8 (standard factor) (van Overbeek FM 1997).

BOWEL INFLAMMATORY INDEX

Measurement of fecal calprotectin

A stool specimen was collected by each subject using a disposable plastic bucket-type device to avoid contact with toilet water and simplify laboratory sampling. Only pre-endoscopy fecal samples were analyzed. Samples were stored at -20°C and thawed at room temperature before testing. The fetal calprotectin (FC) values were analyzed by a commercially available enzyme-linked immunosorbent assay (ELISA) (Calprest, Eurospital Spa, Trieste, Italy). The size of the samples is substantially reduced from 5g to 50-100 mg and FC levels are easily calculated from a standard curve and expressed as µg /g of stools. The cut off level of FC is 95.3 µg/g (Berni Canani R 2004).

Cellobiose/Mannitol Small Intestinal Permeability Ratio

An oral load of two sugars (cellobiose 5 g and mannitol 2 g) was given in 100 mL of water (270 mOsm) to fasting subjects. Urine passed over a 5 hour period was collected and stored at -20°C. Cellobiose and mannitol were measured, as previously described (Troncone R 1994). The final value resulted from the ratio of the recovered percentages of the two sugars, 0.023 is cutoff value.

Computerised bone mineralometry

We evaluated bone quality, measured as amplitude-dependent speed of sound (Ad-SoS) by quantitative ultrasound.

CONCLUSIONS

In the last decade, it has become increasingly evident that CD encompasses a large spectrum of clinical manifestations that range from serious symptomatic forms to completely asymptomatic forms. At the same time, from a histological point of view, it goes from the typical severe lesions of duodenal mucosa to forms characterized by minor degrees of enteropathy.

In clinical practice, by increased diagnostic efficiency of serum CD-related autoantibodies measurements and increased attention for CD than in the past, it is increasingly more common to find patients who have serum positivity for CD autoantibodies but whose duodenal mucosa shows a normal histological appearance. The potential CD is a patient with anti-endomysium antibodies (EMA) and/or anti-human tissue transglutaminase antibodies (anti-TG2) in serum, but a normal mucosal architecture at the intestinal biopsy examination. Good markers of potential CD include subtle pathological alteration such as increased density of intraepithelial lymphocytes expressing γδ TCR, signs of activated mucosal cell-mediated immunity (such as expression of CD25 and B7 by lamina propria mononuclear cells), coeliac-like intestinal antibody pattern.

The patients with minor degrees of enteropathy (potential or early-developing CD) do not fulfill the traditional ESPGHAN diagnostic criteria.

In these case, the diagnosis of disease still rely on a combined approach based of clinical criteria, histology, serology and genetics. Moreover, a revision of the current ESPGHAN criteria might be useful, as has already been frequently suggested, but it should be evidence-based.

From a clinical practical point of view, there are no clear guidelines as to how best to deal with potential CD subjects, because it is little known about the natural history of patients with minor inflammation. More informations are also needed on the features influencing the degree of gluten-dependent inflammation, on the markers predictive of evolution to frank CD and on the health risks they are exposed to, if left on a normal gluten-containing diet. At this propose, we showed that the potential CD is a condition mainly seen in first-degree relatives (28%) and patients with autoimmune disorders (16%). In our cohort anti TG2 titres was lower than that showed by CD with atrophy, in 44% showed a fluctuation of antibodies titres with transient negativity for EMA or anti-TG2 values, while in a 7.4% they returned persistently and completely negative. Finally, duodenal mucosa can remain normal for several years despite the presence of CD associated antibodies. These data are very interesting, but they are not conclusive. A prospective and randomized study with longer follow-up is necessary.

For the first time, in these same patients with potential CD we studied in the duodenal mucosa immunological markers to clarify the early events

leading to enterophaty. In potential CD mucosa T cells are activated and committed Th1 as supported by the increased expression of IL-2, T-bet and IFNγ. Likely, in the early phase of disease IL-10 play a crucial role to prevent the progression towards a complete mucosal damage. In fact, the levels of IL-10 were significantly increased in this group of patients (T0 Marsh) than controls, while in T1 Marsh group they were reduced. In particular the ratio between IL 10 and IFN-γ was increased in group T0 if compared to active coeliac patients and group T1. Moreover, a further data that support the hypothesis that the regulatory mechanisms might downregulate T cell mediate immuno-response in the early phase of CD enterophaty is the increased number of CD4+CD25+FOXP3+ regulatory T cells. The subsequent events leading to failure of these regulatory mechanisms remain to know.

In clinical practice, another particular condition is the CD in patients with selective IgA deficiency. Screening for CD is a special challenge in IgAD since the IgA class CD associated autoantibodies are not produced. In addition, enhanced T cell activation is present in the intestine of IgAD making difficult the differential diagnosis with potential CD. To find markers that could improve the diagnostic accuracy of CD in these subjects, we studied the density of intraepithelial γδ T cells and the deposition of anti TG2 IgM antibodies in duodenal mucosa of IgAD patients. We found that deposited IgM anti TG2 antibodies are present in

only 14% of jejunal specimens of potential CD probably because the low titre/affinity. Of crucial importance for the diagnosis of CD in IgAD patients remains the finding of serum IgG CD-associated autoantibodies.

ACKNOWLEDGMENTS

I would like to thank all the people who help and supported me during my studies leading to the realization of this work.

I should thank Prof. Salvatore Auricchio for the opportunity he gave me to work in his group.

I owe my deepest gratitude to my tutor, Professor Riccardo Troncone, for introducing me to the world of gastrointestinal immunology and for the opportunity he gave me to attend this Ph.D Program and to work in his group. He has supported and encouraged me during all these years. He is also thanked for his critically reviewing the manuscript of this thesis and for their valuable comments.

I would like to thank Dr. Virginia Michela Salvati for her precious help in several experiments and her support in the laboratory. Her knowledge and experience on both clinical and scientific matters and crisp logical thinking have been the driving force of this study. She is not only a colleague but she is also an especially dear friend.

This work would not have been possible without collaboration with several distinguished colleagues. Maura Agnese, Renata Auricchio, Maria Maglio, Francesco Paparo, Luciano Rapacciuolo, Antonella Tosco, Delia Zanzi are gratefully acknowledged for their important and fruitful work.

Finally, my deepest thanks are due to my family, in particular to my dear mother Rosa for her unconditional love, care and belief in me.

REFERENCES

- Abrams JA, Diamond B, Rotterdam H, Green PH. Seronegative celiac disease: increased prevalence with lesser degrees of villous atrophy. Dig Dis Sci 2004; 49:546-550.
- Arranz E, Ferguson A. Intestinal antibody pattern of celiac disease: occurrence in patients with normal jejunal biopsy histology. Gastroenterol 1993; 104:1263-1272.
- Auricchio S, Mazzacca G, Tosi R et al. Coeliac disease as a familiar condition: identification of asymptomatic celiac patients within family groups. Gastroenterol Intern 1988;1:25-31.
- Barone M, Gimigliano A, Castoria G et al. Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease. Gut 2007;56:480-488.
- Beckett CG, Dell'Olio D, Kontakou M et al. Analysis of interleukin-4 and interleukin 10 and their association with the lymphocytic infiltrate in the small intestine of patients with coeliac disease. Gut 1996; 39:818-823.
- van Belzen MJ, Meijer JW, Sandkuiji LA et al. A major non-HLA locus in celiac disease: evidence for linkage to chromosome 19. Gastroenterol 2003;125:1032-1041.
- Berni Canani R, Rapacciuolo L, Romano MT et al. Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice. Dig Liv Dis 2004;36:467-70.

- Bettelli M, Das MP, Howard ED et al. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demostrated by studies of IL-10 and IL-4 deficient and transgenic mice. J Immunol 1998:161:3299-3306.
- Bevan S, Popat S, Braegger CP et al. contribution of the MCH region to the familial risk of celiac disease. J Med Genet 1999;36:687-690.
- Bisikirska B, Colgan J, Luban J et al. TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. J Clin Invest 2005; 115 : 2904-2913.
- Bonamico M, Mariani P, Danesi HM et al. Prevalence and clinical picture of celiac disease in Italian Down syndrome patients: a multicentre study. J Pediatr Gastroenterol Nutr 2001; 33: 139-143.
- Bonamico M, Pasquino AM, Mariani P et al. Prevalence and clinical picture of celiac disease in Turner syndrome. J Clin Endocrinol Matab 2002; 87: 5495-5498.
- Brar P, Know GY, Egbuna II et al. Lack of correlation of degree of villous atrophy with severity of clinical presentation of celiac disease. Dig Liv Dis 2007; 39:26-29.
- Breese EJ, Kumar P, Farthing MJG et al. Interleukin2 and interferon gamma producing cells in the lamina propria in coeliac disease. Dig Dis Sci 1994;39:2243-2248.

- Brunkow ME, Jeffery EW, Hjerrild KA et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001, 27 : 68-73.
- Cataldo F, Marino V, Ventura A, et al. Prevalence and clinical features of selective immunoglobulin A deficiency in celiac disease: an Italian multicentre study. Gut 1998; 42: 362-365.
- Cataldo F and Montalto G. Celiac disease in the developing countries: a new and challenging public health problem World J Gastroenterol 2007;13:2153-2159.
- Carlsson A, Axelsson I, Borulf S, et al. Prevalence of IgA anti gliadin antibodies and IgA anti endomysium antibodies related to celiac disease in children with Down's syndrome. Pediatrics 1998; 101: 272-275.
- Cerwenka A and Swain SL. TGF-beta1: immunosuppressant and viability factor for T lymphocytes. Microbes Infectr 1999; 1:1291-1296.
- Chen Y, Kuchroo VK, Inobe J et al. Regulatory T cell clones induced by oral tolerance : suppression of autoimmune encephalomyelitis. Science 1994;265:1237-1240.
- Ciccocioppo R, Di Sabatino A, Parroni R et al. Cytolytic mechanisms of intraepithelial lymphocytes in coeliac disease. Clin Exp Immunol 2000;120:235-240.
- Ciclitira PJ, Johnson MW, Dewar DH et al. The pathogenesis of coeliac disease.Mol Aspects Med 2005; 26:421-458.

- Corazza GR, Andreani ML, Venturo N, et al. Coeliac disease and alopecia areata: report of a new association. Gastroenterology 1995;109:1333-1337.
- Cunningham-Rundles C, Brandeis WE, Pudifin DJ et al. Autoimmunity in selective IgA deficiency: relationship to anti-bovine protein antibodies, circulating immune complexes and clinical disease. Clin Exp Immunol 1981;45:299-304.
- Cunningham-Rundles C. Physiology of IgA and IgA deficiency. J Clin immunol 2001 21:303-309.
- Daum S, Bauer U, Foss HD et al. Increased expression of mRNA for matrix metalloproteinases-1 and –3 and tissue inhibitor of metalloproteinases-1 in intestinal biopsy speciems from patients with coeliac disease. Gut 1999;44:17-25.
- De Carli M, D'Elios MM, Zanucchi G et al. Human Th1 and Th2 cells: functional properties, regulation of development and role in autoimmunity. Autoimmunity 1994; 18:301-304.
- De Ritis G, Auricchio S, Jones HW et al. In vitro (organ culture) studies of the toxicity of specific A-gliadin peptides in celiac disease. Gastroenterol 1988;94:41-49.
- Dicke WK, Weijer HA and van de Kamer JH. Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. Acta Paediat 1953,42:34-42.
- Dickey W, Hughes DF, McMillan SA. Patients with serum IgA endomysial

antibodies and intact duodenal villi: clinical characteristics and management options. Scand J Gastroenterol 2005;40:1240-1243.

- Donaldson MR, Firth SD, Wimpee H, et al: Correlation of duodenal histology with tissue transglutaminase and endomysial antibody levels in pediatric celiac disease. Clin Gastroenterol Hepatol 2007; 5: 567-573.
- Douglas ID, Gillon J, Logan RFA, et al: Sarcoidosis and coeliac disease: an association. Lancet 1984;2:13-14.
- Earle KE, Tang Q, Zhou X et al. In vitro expanded human CD4+CD25+ regulatory T cells suppress effectror T cell proliferation. Clin Immunol 2005;115:3-9.
- Esposito C, Paparo F, Caputo I et al. Anti-tissue transglutaminase antibodies from coeliac patients inhibit transglutaminase activity both in vitro and in situ. Gut 2002;51:177-181.
- Falchuk ZM, Rogentine GN, Strober W. Predominance of histocompatibility antigen HL A8 in patients with gluten sensitive enteropaty. J Clin Invest. 1972;51:1602-1605.
- Fantuzzi G, Reed DA, Qi M et al. Role of interferon regulatory factror-1 in th regulation of IL18 production and activity. Eur J Immunol 2001; 31:369-375.
- Fasano A. Modulation of intestinal permeability: an innovative method of oral drug delivery for the treatment of inherited and acquired human diseases. Mol Genet Metab 1998;64:12-18.

- Fasano A, Not T, wang W et al. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet 2000;355:1518-1519.
- Fasano A. Intestinal zonulin: open sesame! Gut. 2001 Aug;49:159-62.
- Fasano A, Catassi C. Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. Gastoenterology 2001; 120:636-551.
- Fasano A, Berti I, Gerarduzzi T, et al. Prevalence of Celiac Disease in at-risk and not-at-risk groups in the United States Arch Intern Med 2003; 163:286-292.
- Fasano A. Clinical presentation of Celiac Disease in the Pediatric Population. Gastroenterology 2005; 128:S68-S73.
- Ferguson A, Arranz E, O'Mahony S. Clinical and pathological spectrum of coeliac disease-active, silent, latent, potential. Gut 1993;34:150-1.
- Forsberg G, Hernell O, Melgar S, et al: Paradoxical coexpression of proinflammatory and Down-regulatory cytokines in intestinal T cells in childhood celiac disease. Gastroenterology 2002;123:667-678.
- Forsberg G, Hernell O, Hammarström S, Hammarström ML. Concomitant increase of IL-10 and pro-inflammatory cytokines in intraepithelial lymphocyte subsets in celiac disease. Int Immunol. 2007 ;19:993-1001.
- Friman V, Quiding M, Czerkinsky C et al. Intestinal and circulating antibody-forming cells in IgA-deficient individuals after oral cholera vaccination. Clin Exp Immunol 1994; 95:222-226.

- Fujita T, Kimura Y, Miyamoto M et al. Induction of endogenous IFN alfa and IFN beta genes by IRF1. Nature 1989; 337:270-272.
- Fukaura H, Kent SC, Pietrusewicz MJ et al. induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. J Clin Invest 1996;98:70-77.
- Gianfrani C, Toncone R, La Cava A. Autoimmunity and celiac disease. Mini Rev Med Chem 2008;8:129-134.
- Gilliet M and Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. J Exp Med 2002; 195: 695-704.
- Giorgi PL, Catassi C, Sbarbati A et al. Ultrastructural findings in the jejunal mucosa of children with IgA deficiency. JPGN 1986; 5:892-898.
- Giovannini C, Sanchez M, Straface E et al. Induction of apoptosis in caco-2 cells by wheat gliadin peptides. Toxicology 2000;145:63-71.
- Greco L, Maki M, Di Donato F, Visakorpi IK. Epidemiology of coeliac disease in Europe and the Mediterranean area: a summary report on multicentre study by the European Society of Paediatric Gastroenterology and Nutrition. In Auricchio S, Visakorpi JK (eds): Common Food Intolerances. Vol 1: Epidemiology of Coeliac Disease. Basel, Karger, 1992, pp 25-44.
- Greco L, Babron MC, Corazza GR et al. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. Ann Hum Genet 2001;65:35-41.

- Greco L, Romino R, Coto I et al. The first large population based twin study of coeliac disease. Gut 2002; 50:624-628.
- Green PH and Jabri B. Coeliac disease. Lancet 2003;362:1418-9.
- Groux H. Type 1 T-regulatory cells: their role in the control of immune responses.Transplantation 2003; 75:8S-12S.
- van Heel DA, Franke L, Hunt KA et al. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. Nat Genet 2007; 39:827-829.
- Hill ID. What are the sensitivity and specificy of serologic tests for celiac disease? Do sensitivity and specificy vary in different populations. Gastroenterol 2005;128: S25-S32.
- Ho IC and Glimcher LH. Transcription: tantalizing times for T cells. Cell 2002; 109:S109-S120.
- Hoffenberg EJ, Bao F, Eisenbarth GS et al. Transglutaminase antibodies in children with a genetic risk for celiac disease. J Pediatr 2000;137:356-360.
- Hüe S, Mention JJ, Monteiro RC et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 2004;21:367-377.
- Huibregtse IL, van Lent AU, van Deventer SJH. Immunopathogenesis of IBD : insufficient suppressor function in the gut? Gut 2007;56:584-592.
- Hunt KA, Zhernahova A, Turner G et al. Newly identified genetic risk variants for celiac disease related to the immune response. Nat Gen 2008;40:385-402.

- Iltanen S, Holm K, Partanen J et al. Increased density of jejunal gammadelta+ T cells in patients having normal mucosa--marker of operative autoimmune mechanisms? Autoimmunity 1999; 29:179-187.
- Jabri B, de Serre NP, Cellier C et al. Selective expansion of intraepithelial lymphocytes expressing the HLA-E-specific natural killer receptor CD94 in celiac disease. Gastoenterol 2000;118:867-879.
- Jabri B, Kasarda DD, Green PH. Innate and adaptive immunity: the yin and yang of coeliac disease. Immunol Rev 2005;206:219-231.
- Jabri B, Ebert E. Human CD8+ intraepithelial lymphocytes: a unique model to study the regulation of effector cytotoxic T lymphocytes in tissue. Immunol Rev 2007;215:202-214.
- Jacob CM, Pastorino AC, Fahl K et al. Autoimmunity in IgA deficiency: revisiting the role of IgA as a silent housekeeper. J Clin Immunol 2008; 28 Suppl 1:S56-S61.
- Jarvinen TT, Kaukinen K, Laurila K, et al: Intraepithelial lymphocytes in celiac disease. Am J Gastroenterol 2003; 98: 1332-1337.
- Jarvinen TT, Collin P, Rasmussen M, et al: Villous tip intraepithelial lymphocytes as markers of early-stage celiac. Scand J Gastroenterol 2004; 39: 428-33.
- Kagnoff MF. Celiac disease: pathogenesis of a model immunogenetic disease. J Clin Invest 2007;117:41-49.

- Kamijo R, Harada H, Matsuyama T et al. Requirement for transcripton factor IRF-1 in NO synthase induction in macrophages. Science 1994;263:1612-1615.
- Karell K, Louka AS, Moodie SJ et al. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European genetics cluster on celiac disease. Hum Immunol 2003; 64:469-477.
- Kaukinen K, Maki M, Partanen J et al. Celiac disease without atrophy. Dig Dis Sci 2001; 46: 879-887.
- Kaukinen K, Partanen J, Maki M, Collin P. HLA-DQ typing in the diagnosis of coeliac disease. Am J Gastroenterol 2002; 97:695-699.
- Kaukinen K, Peraaho M, Korpornay-Szabo IR et al. Small-bowel mucosal transglutaminasi2–specific-IgA-deposits in coeliac disease without villous atrophy: a prospective and randomized clinical study. Scand J Gastroenterol 2005; 40: 564-572.
- Kaukinen K, Mäki M, Collin P. Immunohistochemical features in antiendomysium positive patients with normal villous architecture. Am J Gastroenterol 2006;101:675-676.
- Kim J, Woods A, Becker-Dunn E, Bottomly K. Distinct functional phenotypes of cloned Ia-restricted Helper T cells. J Exp Med 1985;162:188-200.
- Kimura T, Kadokawa Y, Harada H et al. Essential and non-redundant roles of p48(ISGF3 gamma) and IRF1 in both type I and type II interferon response, as revealed by gene targeting studies. Genes Cells 1996;1:115-124.

- Klemola T. Deficiency of immunoglobulin A. Ann Clin Res 1987;19:248-257.
- Klemola T. Immunohistochemical findings in the intestine of IgA-deficient persons: number of intraepithelial T lymphocytes is increased. JPGN 1988; 7:537-543
- Klemola T, Savilahti E, Arato A et al. Immunohistochemical findings in jejunal specimens from patients with IgA deficiency. Gut 1995;37:519-523.
- Koistinen J. Selective IgA deficiency in blood donors. Vox Sang 1975;29:192-202.
- Kontakou M, Przemioslo RT, Sturgess RP et al. Expression of tumour necrosis factror-α, interleukin-6 and interleukin-2 mRNA in the jejunum of patients with coeliac disease. Scand J Gastroenterol 1995;, 30:456-463.
- Korponay-Szabo IR, Dahlbom I, Laurila K et al. Elevation of IgG antibodies against tissue transglutaminase as a diagnostic tool for coeliac disease in selective IgA deficiency.Gut 2003;52:1567-1571.
- Korponay-Szabo IR, Halttunen T, Szalai Z et al. In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies. Gut 2004;53:641-8.
- Kuhn R, Lohler J, Rennick D et al. Inteleukin-10 deficient mice develop chronic enterocolitis. Cell 1993;75:263-274.
- Lahat N, Shapiro S, Karban A et al. Cytokine profile in coeliac disease. Scand J Immunol 1999; 49:441-446.
- Levings MK, Sangregorio R, Sartirana C et al. Human CD25+CD4+ T

suppressor cell clones produce transforming growth factor beta but not interleukin 10, an are distinct from type 1 T regulatory cells. J Exp Med 2002; 196:1335-1346.

- Levings MK, Gregori S, Tresoldi E et al. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. Blood 2005; 105: 1162-1169.
- Li B, Yang P, Chu L, Zhou H, Huang X, Zhu L, Kijlstra A: T-bet expression in the iris and spleen parallels disease expression during endotoxin-induced uveitis. Graefes Arch Clin Exp Ophthalmol 2006.
- Lionetti P, Pazzaglia A, Moriondo M et al. Differing patterns of trasformino growth factor-β expression in normal intestinal mucosa and in active celiac disease. J Pediatr Gastroenterol Nutr 1999; 29:308-313.
- Liu J, Juo SH, Holopainen P et al. Genome wide linkage analysis of celiac disease in Finnish families. Am J Hum Genet 2002;70:51-59.
- Liu W, Putnam AL, Xu-Yu Z et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006 ; 203 :1701-1711.
- Lo W, Sano K, Lebwohl B et al. Changing presentation of adult coeliac disease. Dig Dis Sci 2003; 48:395-398.
- Louka AS, Nilsson S, Olsson M et al. HLA in celiac disease families: a novel test of risk modification by the "other" haplotype when at the least one DQA1*05-DQB1*02 haplotype is carried. Tissue Antigens 2002;60:147-154.

- MacDonald TT and Monteleone G. IL12 and Th1 immune responses in human Peyer's patches. Trends Immunol 2001; 22:244-247.
- MacDonald TT. The mucosal immune system. Parasite Immunol 2003; 25:235-246.
- Maiuri L, Ciacci C, Auricchio S et al. Interleukin 15 mediates ephitelial changes in celiac disease. Gastroenterol 2000;119:996-1006.
- Maiuri L, Ciacci C, Raia V, et al: FAS engagement drives apoptosis of enterocytes of coeliac patients. Gut. 2001;48:418-424.
- Maiuri L, Ciacci C, Ricciardelli I et al. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet 2003;362:30-37.
- Maki M, Holm K, Collin P, Savilahti E. Increase in gamma/delta T cell receptor bearing lymphocytes in normal small bowel mucosa in latent coeliac disease. Gut 1991;32:1412-1414.
- Maki M, Holm K, Ascher H et al. Factors affecting clinical presentation of celiac disease: role of type and amount of gluten containing cereals in the diet. In Common Food Intolerances 1: Epidemiology of Coeliac Disease. Edited by Auricchio S, Visakorpi JK. Basel: Karger; 1992:76-82.
- Maki M, Mustalahti K, Kokkonen J, et al: Prevalance of celiac disease among children in Finland. N Engl J Med 2003;348:2517-2524.
- Mantovani V, Corazza GR, Bragliani M et al. Asp57-negative HLADQβ chain and DQA1*0501 allele are essential for the onset of DQw2-positive and

DQw2-negative coeliac disease. Clin Exp Immunol 1993;91:153-156.

- Mari T, Zullo A, Hassan C et al. genetic association between factor V Leiden and coeliac disease. Gut 2006;55:1677-1678.
- Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ("celiac sprue"). Gastroenterology 1992;102:330-354.
- Marzari R, Sblattero D, Florian F et al. Molecular dissection of the tissue transglutaminase autoantibody response in celiac disease. J Immunol 2001;166:4170-4176.
- Mayer M, Greco L, Troncone R, et al: Early prediction of relapse during gluten challenge in childhood coeliac disease. J Pediatr Gastroenterol Nutr 1989;8:474-479.
- Mazzarella G, MacDonald TT, Salvati VM, et al: Constitutive activation of the signal transducer and activator of trascription pathway in celiac disease lesions. Am J Pathol 2003;162:1845-1855.
- Meresse B, Curran SA, Ciszewski C et al. Reprogramming of CTLs into natural killer-like cells in celiac disease. J Exp Med 2006; 203:1343-55.
- Molberg O, Mcadam SN, Körner R et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut derived T cells in celiac disease. Nat Med 1998;4:713-717.
- Monsuur AJ and Wijmenga C. Understanding the molecular basis of celiac sidease: what genetic studies reveal. Ann Med 2006; 38:578-591.

- Monteleone G, Pender SL, Alstead E, et al: Role of interferon alpha in promoting T helper cell type 1 responses in the small intestine in coeliac disease. Gut 2001;48:425-429.
- Monteleone I, Monteleone G, Del Vecchio Blanco G et al. Regulation of the T helper cell type 1 transcription factor T-bet in coeliac disease mucosa. Gut 2004; 53:1090-1095.
- Mosmann TR, Cherwinski H, Bond MW et al. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-2357.
- Mulder CJJ, Ytgat GNJ, Groenland F et al. Combined coeliac disease and thyroid disease: a study of 17 cases. J Clin Nutr Gastroenterol 1988;3:89-92.
- Mounsuur AJ, de Bakker PI, Alizadeh BZ et al. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. Nat Genet 2005;37:1341-1344.
- Naluai AT, Nilsson S, Gudjonsdottir AH et al. Genome wide linkage analysis of Scandinavian affected sibpairs supports presence of susceptibility loci for celiac disease on chromosome 56 and 11. Eur J Hum Genet 2001;9:938-944.
- Natvig IB, Johansen FE, Nordeng TW et al. Mechanism for enhanced external transfer of dimeric IgA over pentameric IgM: studies of diffusion, binding to the human polymeric Ig receptor, and epithelial transcytosis. J Immunol 1997; 159:4330-4340.

- Nilsen EM, Jahnsen FL, Lundin KEA et al. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. Gastroenterol 1998;115:551-563.
- Nilssen DE, Aukrust P, Frøland SS et al. Duodenal intraepithelial gamma/delta T cells and soluble CD8, neopterin, and beta 2-microglobulin in serum of IgA-deficient subjects with or without IgG subclass deficiency. Clin Exp Immunol 1993; 94:91-98.
- Nistico L, Fagnani C, Coto I et al. Concordance, disease progression, and heritability of coeliac disease in Italian twins. Gut 2006;55:803-808.
- Not T, Faleschini E, Tommasini A, et al. Celiac disease in patients with sporadic and inherited cardiomyopathies and in their relatives. Eur Heart J 2003; 24:1455-1461.
- Oberhuber G, Granditsch G, Vogelsang H et al . The histopathology of coeliac disease: time for a standardized report scheme for pathologists. Eur J Gastroenterol Hepatol 1999; 11: 1185-1194.
- Ozaslan E, Akkorlu S, Eskioaylu E et al: Prevalence of silent celiac disease in patients with dyspepsia. Dig Dis Sci 2007, 52:692-697.
- Paparo F, Petrone E, Tosco A, et al: Clinical, HLA, and small bowel immunohistochemical features of children with positive serum antiendomysium antibodies and architecturally normal small intestinal mucosa. Am J Gastroenterol 2005;100:2294-8.

- Pestka S, Krause CD, Sarkar D et al. Interleukin-10 and related cytokines and receptors. Annu Rev Immunol 2004; 22: 929-979.
- Picarelli A, Maiuri L, Frate A et al. Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease. Lancet 1996;348:1065-1067.
- Pignata C, Budillon G, Monaco G et al. Jejunal bacterial overgrowth and intestinal permeability in children with immunodeficiency syndromes. Gut 1990:31:879-882.
- Powrie F, Leach MW, Mauze S et al. inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi T cells. Immunity 1994;1:553-562.
- Ravikumara M Tuthill DP and Jenkins HR: The changing clinical presentation of celiac disease. Arch Dis Child 2006, 91:969-971.
- Reunala T, Salmi J, Karvonen J. Dermatitis herpetiformis and coeliac disease associated with Addison's disease. Arch Dermatol 1987;123:930-932.
- Romanos J, Barisani D, Trynka G et al. Six new celiac disease loci replicated in an Italian population confirm association to celiac disease. J Med Gen 2008 Sep 19. (Epub ahead of print).
- Roncarolo MG, Bacchetta R, Bordignon C et al. Type 1 T regulatory cells. Immunol Rev 2001;182:68-79.
- Rostami K, Kerckhaert J, Tiemessen R, et al: Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical

practice. Am J Gastroenterol 1999; 94: 888-894.

- Rostom A, Dube C, Cranney A, et al. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. Gastroenterol 2005; 128:S38-S46.
- Sacchetti L, Tinto N, Calcagno G et al. Multiplex PCR typing of the three most frequent HLA alleles in celiac disease. Clin Chim Acta. 2001 20;310:205-7.
- Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004 ; 22 : 531-562.
- Salmi TT, Collin P, Jarvinen O et al. Immunoglubulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease. Aliment Pharmacol Ther 2006;24:541-552.
- Salvati VM, Troncone R, Borrelli M et al. mRNA transcripts for γ interferon, but also for IL10, are increased in the small intestinal mucosa of untreated coleica patients. Immunology Letters 1999;69:104.
- Salvati VM, Bajaj-Elliott M, Poulsom R, et al: Keratinocyte growth factor and coeliac disease. Gut 2001;49:176-181.
- Salvati VM, MacDonald TT, Bajaj-Elliott M et al. Interleukin 18 and associated markers of T helper cell type 1 activity in celiac disease. Gut 2002;50:186-190.
- Salvati VM, MacDonald TT, Del Vecchio Blanco G et al. Enhanced expression of interferon regulatory factror-1 in the mucosa of children with celiac disease. Pediatric Research 2003;54:312-318.

- Samoilova EB, Horton JL, Chen Y et al. Acceleration of experimental autoimmune encephalomyelitis in interleukin-10 deficient mice: roles of interleukin-10 in disease progression and recovery. Cell Immunol 1998;188:118-124.
- Sanderson IR and Walker WA. Mucosal barrier: an overview. In: Ogra PL, Mestecky J, Lamm ME,Strober W, Bienenstock J and McGhee JR (eds.). Mucosal Immunology. Academic press, San Diego, 1999, pp 5-17.
- Savilahti E. IgA deficiency in children. Immunoglobulin-containing cells in the intestinal mucosa, immunoglobulins in secretions and serum IgA levels. Clin Exp Immunol 1973;13;:395-406.
- Savilahti E, Simell O, Koskimes S, et al. Coeliac disease in insulin- dependent diabetes mellitus. J Pediatr 1986;108:690-693.
- Savilahti E, Klemola T, Carlsson B et al. Inadequacy of mucosal IgM antibodies in selective IgA deficiency: excretion of attenuated polio viruses is prolonged. J Clin Immunol 1988;8:89-94.
- Sategna-Guidetti C, Sategna-Guidetti C, PulitanÃ³ R, Grosso S, Ferfoglia G: Serum IgA antiendomysium antibody titers as a marker of intestinal involvement and diet compliance in adult celiac sprue. J Clin Gastroenterol 1993; 17:123-127.
- Schulzke JD, Schulzke I, Fromm M, Riecken EO. Epithelial barrier and ion trasport in coeliac disease: electrical measurements on intestinal aspiration biopsy specimens. Gut 1995;37:777-782.

- Schulzke JD, Bentzel CJ, Schulzke I et al. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. Pediatr Res 1998;43:435-441.
- Seddiki N, Santner-Nanan B, Martinson J et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006 ; 203 1693-1700.
- Seder RA. High-dose IL2 and IL15 enhance the in vitro priming of naïve CD4+ cells for IFN gamma but have differential effects on priming for IL4. J Immunol 1996;156:2413-2422.
- Setoguchi R, Hori S, Takahashi T et al. Homeostatic maintenance of natural Foxp3+CD25+CD4+ regulatory T cells interleukin (IL)-2 and indution of autoimmune disease by IL-2 neutralization. J Exp Med 2005;201:723-735.
- Shan L Molberg O, Parrot I et al. Structural basis for gluten intolerance in celiac sprue. Science 2002;297:2275-2279.
- Shan L et al. Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. J Proteome Res 2005;4:1732-1741.
- Shevach EM. Regulatory/suppressor T cells in health and disease. Arthritis Rheum 2004 ; 50 : 2721-2724.
- Siegmund B, Sennello JA, Lehr HA, Senaldi G, Dinarello CA, Fantuzzi G: Interferon regulatory factor-1 as a protective gene in intestinal inflammation:

role of TCR g/d T cells and interleukin-18-binding protein. Eur J Immunol 2004; 34: 2356–2364.

- Smecuol E, Sugai E, Niveloni S et al. Permeability, zonulin production, and enteropathy in dermatitis herpetiformis. Clin Gastroenterol Hepatol 2005;3:335-341.
- Sollid LM, Markussen G, EK J et al. Evidence for a primary association of celiac disease to a particular HLA DQ alpha/beta heterodimer. J Exp Med 1989;91:153-156.
- Sollid LM, Molberg O, Mcadam S et al. Autoantobodies in coeliac disease: tissue transglutaminase-guilt by association? Gut 1997;41:851-852.
- Sollid LM, Jabri B. Is celiac disease an autoimmune disorder? Curr Opin Immunol 2005;17:595-600.
- Spencer J, Isaacson PG, MacDonald TT, et al. Gamma/delta cells and the diagnosis of celiac disease. Clin Exp Immunol 1991;85:109-113.
- Steinbrink K, Jonuleit H, Müller G et al. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood 1999; 93: 1634-1642.
- Stene-Larsen G, Mosvold J, Ly B. Selective vitarnin B12 malabsorption in adult coeliac disease: report on three cases with associated autoim- mune diseases. Scand J Gastroenterol 1988;23:1105-1108.
- Stenhammar L, Ljunggren CG. Thrombocytopenic purpura and coeliac disease. Acta Paediatr Scand 1988;77:764-766.

- Strobl H, Knapp W. TGF-beta1 regulation of dendritic cells. Microbes Infect 1999; 1:1283-1290.
- Strokes PL, Asquith P, Holmes GK et al. Histocompatibility antigens associated with adult celiac disease. Lancet 1972;2:162-164.
- Szabo SJ, Kim ST, Costa GL et al. A novel transcription factor, T-bet directs Th1 lineage commitment. Cell 2000;100: 655-669.
- Taniguchi T, Ogasawara K, Takaoka A et al. IRF family of transcription factors as regulators of host defence. Annu Rev Immunol 2001;19:623-655.
- Tosco A, Maglio M, Paparo F et al. Immunoglobulin A anti-tissue transglutaminase antibody deposits in the small intestinal mucosa of children with no villous atrophy. J Pediatr Gastroenterol Nutr 2008; 47: 293-298.
- Troncone R, Caputo N, Florio G et al. Increased intestinal sugar permeability after challenge in children with cow's milk allergy or intolerance. Allergy 1994;49:142-6.
- Troncone R, Greco L, Mayer M et al: Latent and potential coeliac disease. Acta Paediatric Suppl 1996, 412: 10-14.
- Troncone R, Gianfrani C, Mazzarella G et al. The majority of gliadin specific T cell clones from the coeliac small intestinal mucosa produce both interferon gamma and IL4. Dig Dis Sci 1998;43:156-161.
- Troncone R, Maurano F, Rossi M et al. IgA antibodies to tissue transglutaminase: An effective diagnostic test for celiac disease. J Pediatr. 1999;134:166-71.

- Tursi A, Brandimarte G, Giorgetti GM et al. Endoscopic features of celiac disease in adults and their correlation with age, histology damage, and clinical form of the disease. Endoscopy 2002; 34:787-792.
- Tursi A, Brandimarte G, Giorgetti GM: Prevalence of antitissue transglutaminase antibodies in different degrees of intestinal damage in celiac disease. J Clin Gastroenterol 2003;36:219-221.
- Uil JJ, van Elburg RM, van Overbeek FM et al. Follow-up of treated coeliac patients: sugar absorption test and intestinal biopsies compared. Eur J Gastroenterol Hepatol 1996;8:219-223.
- Vader LW, de Ru A, van der Wal Y et al. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. J Exp Med 2002:195:643-9.
- Vader W, Kooy Y, Van Veelen P et al. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. Gastroenterol 2002;122:1729-1737.
- Valletta E, Bertini M, Piccoli R et al. Latent Celiac or Low-Gluten- Containing Diet? J Pediatric Gastroenterol Nutr 2002; 34:91-92.
- Vieira PL, Christensen JR, Minaee S et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol 2004; 172: 5986-5993.
- Villalta D, Alessio MG, Tampoia M, et al Testing for IgG class antibodies in celiac disease patients with selective IgA deficiency. A comparison of the

diagnostic accuracy of 9 IgG anti-tissue transglutaminase, 1 IgG anti-gliadin and 1 IgG anti-deaminated gliadin peptide antibody assays. Clin Chimica Acta 2007;382:95-99.

- Vorechovsky, V, Cullen M, Carrington M et al. Fine mapping of IGAD1 in IgA deficiency and common variable immunodeficiency: identification and characterization of haplotypes shared by affected members of 101 multiple-case families. J Immunol 2000 ;164:4408-4416.
- Wahnschaffe U, Ullrich R Riecken EO et al. Celiac disease like abnormalities in a subgroup of patients with irritable bowel syndrome. Gastroenterol 2001; 121: 1329.1338.
- Walker MR, Kasprowicz DJ, Gersuk VH et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003; 112:1437-1443.
- Walker-Smith JA. Management of infantile gastroenteritis. Arch Dis Child. 1990;65:917-918.
- Wang W, Uzzau S, Goldblum SE et al. Human zonulin, a potential modulator of intestinal thigh junctions. J Cell Sci 2000;113:4435-4440.
- Weizman Z, Ben–Zion YZ, Binsztok M, et al. Correlation of clinical characteristics and small bowel histopathology in celiac disease. J Pediatr Gastroenterol Nutr 1997; 24: 555-558.
- Westerholm-Ormio M, Garioch J, Ketola I et al. Inflammatory cytokines in small intestinal mucosa of patients with potential coeliac disease. Clin Exp

Immunol 2002; 128: 94-101.

- Wieser H. Relation between gliadin structure and coeliac toxicity. Acta Paediatr. Suppl 1996;412:3-9.
- Wolters VM and Wijmenga C. genetic background of celiac disease and its clinical implications. Am J Gastroenterol 2008;103:190-195.
- Working Group of ESPGAN. Revised criteria for diagnosis of celiac disease. Arch Dis Child 1990; 65: 909-11.
- Zhong F, McCombs CC, Olson JM et al. An autosomal screen for genes that predispone to celiac disease in the western counties of Ireland. Nat Genet 1996;14:329-333.