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Identification of gliadin peptides immunogenic for celiac patients

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BACKGROUND

Celiac Disease

Celiac Disease (CD) is a gluten-sensitive enteropathy which occurs in 1% of Caucasian populations. This multifactorial disorder arises, in genetically susceptible individuals, following exposure to dietary wheat gluten, and similar proteins (prolamins), present in rye and barley. Susceptibility to develop celiac disease is strongly influenced by inherited factors, as confirmed by high prevalence (~10%) among first-degree relatives of CD patients (1).

Main genetic factors involved in celiac disease are the Human Leukocyte Antigen (HLA) Class II genes that encoded HLA-DQ2 (2) and HLA-DQ8 heterodimers (3). The strong genetic influence related to celiac disease is also supported by a high concordance rate (75%) in monozygotic twins, while the concordance rate between dizygotic twins is at the 11%. (4,5). Despite the frequency of celiac HLA class II in general population is ~30%, only 1% develops celiac disease (6). These features suggest that these particular HLA molecules are necessary but

not sufficient for development of celiac disease, and other genetic or environmental factors probably are involved in disease (7).

Clinical aspects and therapy

In susceptible individuals, the ingestion of gluten activates an immune cascade responsible for lymphocytic infiltration of proximal part of the small bowel (8), and for destruction of the intestinal mucosa epithelium (9).

The intestinal damage can range from very mild degree, identified in atypical or silent celiacs, with little or absent histological intestinal lesions, until high or total degree of villous atrophy. In this context an important support to clinical practice and diagnosis was given by Marsh studies. Marsh classification categorized small intestinal damage from normal to complete atrophy, and made possible the interpretation of gluten induced damage (10).

From the clinical point of view, celiac disease presents with different forms (11). In the *classical* or *typical form*, the ingestion of gluten

induced an enteropathy presenting with signs of malabsorption (such as diarrhoea or malnutrition) or malabsorption syndrome (weight loss, steatorrhoea and oedema secondary to hypoalbuminemia). From histological point of view, the disease is characterized by villous atrophy and crypt hyperplasia occurring after introduction of gluten.

In addition to the classical form, most common in childhood, the pathology may occur as "*atypical*". These patients have not weight loss, but present any of the following symptoms such as gastrointestinal symptoms, including symptoms suggestive of irritable bowel syndrome and liver dysfunction; extraintestinal manifestations, such as metabolic disease/symptoms (failure to thrive, thyroid dysfunction); neurologic findings, including depression and gluten ataxia; reproductive disease, including abnormalities in menarche and menopause; oral/cutaneous disease, including dermatitis herpetiformis and skeletal findings (12, 13). Sometimes, the disease may be "*silent*", with no symptoms, but in the presence of small intestine atrophy. In most cases of this category have been identified by screening programs involving, apparently, healthy subjects o first degree relatives of patients.

In addition, there are genetically predisposed children, who have anti-tissue transglutaminase auto-antibodies (tTG2), the serological marker of the disease, but a normal, not damaged, small bowel mucosa (13). Some of these individuals, called "*potential celiac*", will develop the disease over the time (14, 15).

Gluten-free diet is, currently, the only valid treatment of celiac disease (16). The gluten-free diet consist in an alimentary regimen avoiding wheat, rye, barley, and all related cereals including spelt (a wheat variant). Among cereals Oats seems to be no toxic in a great part of celiac, despite that, it is not recommended, since not tolerated by subgroup of patients. For this reason its use is under debate (16, 17).

So far it's not identified any safe threshold for gluten intake in celiac. (16). The guidelines of *Codex Alimentarius* limits the amount of gluten in products for the diets of celiac subjects at less than 20 ppm (12).

Alternative therapies

The gluten free diet allows the restoration of normal intestinal morphology and function, but does not guarantee the recovery of tolerance to these grains, even after many years. Furthermore, gluten free diet is more expensive, and for some patients it has a reduced palatability, responsible of poor compliance.

For all these reasons, recently great research efforts were made to identify a pharmacological therapy alternative to the gluten-free diet (GFD), aimed to recover the immune tolerance to ingested gluten, and to improve the patients' quality life (18).

The identification of a new alternative therapy could be important for a part of treated celiac patients to whom the GFD fails. In these patients the symptoms remained, or reappear, even having gluten free diet. This may be related to inadequate adherence to the diet, or to unintentional ingestion of gluten caused by contamination. A part of these patients are represented by refractory celiac in which the exclusion of gluten from dietary is inefficacious (refractory CD), and serious complications can occur (19, 20).

Several of these studies are address to search ancient wheats devoid of immunogenic sequences, or other naturally non toxic cereals.

However a great part of them were addressed to identify new strategies to detoxify gluten. It has been widely shown that gliadin peptides are characterized by high content in proline that makes it highly resistant to gastrointestinal digestion (21).

For this reason same research efforts were oriented to digest gluten fragments by oral supplementation of proteolytic enzyme (21, 22) in order to promote complete digestion of cereal proteins, and thus destroy immune-stimulatory T-cell epitopes. One drug based on this approach, currently under clinical trial is the enzyme formulation ALV003. This drug seems to be well tolerated by celiac patients, and adverse reactions were not identified. However it's not possible to be sure about long term effects yet (23). Furthermore currently under clinical trials is AN-PEP a prolyl oligopeptidases derived from *Aspergillus niger*. This enzyme is able to cleave gluten. Currently AN-PEP appears to be tolerated, in celiac patients, but a further investigation is required (24, 25).

Another approach is based on proteolytic digestion, and focused on pre-treatment of wheat with lactobacilli able to proteolyze proline-rich gluten peptides (26). In vitro studies confirmed the degradation of toxic epitopes (27).

Another strategy addressed to transamidation of wheat flour it has been implemented. Using this approach the stimulatory activity of gliadin is abolished, while an appropriate amine group donor blocked gliadin immunotoxicity preserving the integrity of the structure (28).

Furthermore studies are instead addressed to reduce intestinal permeability that is a typical feature of celiac disease. It has been demonstrated that this alteration is due to up-regulation of zonulin that causes an opening of tight junctions and a rapid increase in intestinal permeability (29). The use of an inhibitor of zonulin such may have an important effect on gliadin access to GALT. AT-1001 peptide has been synthesized to prevent the up-regulation of zonulin (30).

Despite all these approaches, the peptide-based immunotherapy represents one of the most interesting hopes for the future. This kind of immunotherapy, already used in some allergies, aims to induce

tolerization of reactive T cells and consequently control of inflammation (31). Recently it has been given a great attention to identification of gliadin peptides. Anderson and co-workers, in fact in 2000 used a new *in vivo* system to identify gluten specific T cells, that overcomes the problems related with the used of intestinal T cells. In particular this technique is based on administration of wheat bread for few days (3 days) to celiac patients on GFD, followed by the use of IFN-γ EliSpot Assay for detection of intestinal gluten specific $CD4^{+}$T lymphocytes in peripheral blood. In fact specific gluten T cell lines can be isolated from intestinal biopsies cultured *in vitro*, furthermore these specific T cells, can be found in peripheral blood 3 or 6 days following oral challenge with gluten, in celiac patients on gluten free diet (GFD) (32).

Using this approach, J.A. Tye-Din and co-workers, following a challenge with different cereals, screened a large library of 20-mer epitopes derived from different prolamines. Thanks to this new approach it was provided a comprehensive understanding of the T cell immunostimulatory gluten peptides in HLA-DQ2.5 adults with CD (33). Using both approaches, different studies have identified peptides with

common immunogenic sequence. This peculiarity emphasized a convergence between *in vitro* and *in vivo* findings.

Gluten: main trigger of the disease

As mentioned above, gluten is the main environmental factor involved in celiac disease, and it is the main storage protein of seed in many cereals, including wheat, barley, and rye. Gluten is composed by hundreds of proteins presents as mono-oligomeric o polymers linked by disulphide bonds.

Gluten is characterized by high content of proline, which makes it resistant to gastrointestinal enzyme degradation. Due to this chemical feature following ingestion of gluten, gut lumen, and, in particular the intestinal mucosa come in contact with long fragments (10-50 residues) of gluten, that are crucial for inflammatory activations. Furthermore these long fragments are substrate for the enzyme tissue transglutaminase (tTG2), which converts glutamine residues into glutamate. This process, known as deamidation, increases the gluten

negative residues, and, increases the ability of gluten to bind HLA DQ2 or DQ8, when they are presented by antigen-presenting cells (APC) (34).

Gluten is composed by a mixture of water insoluble proteins gliadins and glutenins. Gliadins are monomeric alcohol insoluble proteins (27-70 KDa), and are rich in prolamine. Gliadins are divided, according their electrophoresis mobility, into three families: α/β-, γ- and ω- gliadins that even so have similar primary structure. Glutenins are divided into low molecular (LMW 30-60 kDa) and high molecular weight (HMW, 80-120 kDa) (35). The great heterogeneity found in gliadin and glutenin is responsible of presence of many distinct T cell epitopes (34).

Genetics of Celiac Disease: HLA and non HLA genes

From the pathogenic point of view, celiac disease is a multifactorial disease, determined by the interaction of genetic and environmental factors. In fact, it only develops in genetically susceptible individuals, which express HLA class II molecules DQ2 and/or DQ8. Over 90% of patients with celiac disease express HLA DQ2 heterodimer encoded by allele HLA-DQA1*05 and by allele HLA-DQB1*02 (36), while the

remaining express the heterodimer DQ8, encoded by allele DQA1*03 and by allele DQB1*0302 (37).

The fact that HLA DQ2/DQ8 alleles are very common in the healthy population suggests that these molecules are necessary but not sufficient for the development of the disease. In fact, the frequency of HLA class II molecules associated to risk of celiac disease is approximately 30% in Caucasians, but only 1-2% of population will develop the disease (38). It is widely demonstrated that HLA class II molecules account for about 35% of the genetic risk, and, at the present there are numerous other non-HLA genes candidate as factors predisposing to the development of celiac disease (39).

In the identification of non HLA genes, the role of genome wide association studies (GWAS) is crucial. The genome wide association studies, using SNP (tag single nucleotide polymorphism) technique, identified a large number of genes implicated in celiac disease, and in other auto-immune disease. In particular, on 40 genomic regions, 64 genes have been identified as candidate genes. These regions explain only 5% of the genetic inheritance (39). Almost all genes found

associated with celiac disease are involved in the immunological cascade, in particular those coding for chemokine receptor activity, T cell activation etc. One important region identified corresponds to the locus present on chromosome 4q27, in which were identified genes encoding interleukin-2 (IL-2), and IL-21, as well as other genes (40). Both these cytokines are important for proliferation and maturation of T-lymphocyte. In another locus identified on chromosome 3q25, were identified genes encoding IL-12, while genes found on chromosome 2q12, expressed IL18-receptor. Both these two cytokines are involved in the regulation of IFN-γ production (40, 41).

All these findings confirmed that CD is a T cell-mediated immune disorder (34).

Immunopathogenesis of CD

As mentioned above, the main environmental factor that triggers celiac disease is gluten. In the celiac gut, the partially digested gluten (that is highly resistant to intestinal degradation) passes through the epithelial

barrier via epithelial transcytosis, or by an increased epithelial tight junctional permeability, that represents a disease hallmark (42).

In the lamina propria gluten peptides are processed by APC, and presented by the surface HLA class II DQ2 or DQ8 molecules, after a specific reaction of deamidation by tTG2.

tTG2 is an ubiquitously enzyme expressed by almost all cells types. It is, usually, presents in enzymatically inactive form in the intracellular compartment, where binds GTP molecules. In particular situation, as for example under mechanical or inflammatory stress, and in reducing environment, tTG2 is released in the extracellular matrix, where in presence of low levels of GTP, and high levels of $Ca2^{+}$, acquires an open enzymatically active form (43).

tTG2 catalyzes different cross-linking reaction. In particular, catalyzes a covalent process of transamidation, that is an irreversible cross-linking of a protein, such as gluten, with a glutamine residue (glutamine donor), to a second protein with a lysine residue (glutamine acceptor). This process generates an irreversible isopeptidyl bond. Alternatively, in absence of lysine residue, and at relatively low pH (<7), TG2 converts

glutamine (neutral) in glutamic acid (negatively) residue. TG2 activity is highly site-specific because deamidate only glutamine residue present in QXP site (Q is a glutamine, P is a prolin and X is any amminoacid). This process of deamidation create negatively charged residue glutamic acid, that facilitates the binding of gluten to HLA-DQ2 and HLA-DQ8, present on APCs, who bind preferentially peptides that contain amino acid with negative charge (44,45).

Following deamidation, gluten peptides acquire negative charged, and bind strongly to the specific HLA molecules (46). The subsequent adaptive response is mediated by $CD4^{+}$T cells that recognise the HLA gluten complex, and produced pro-inflammatory cytokines mainly IFN-γ, responsible for the intestinal damage. This cytokine is one of the main mediators of the pro-inflammatory cascade, and its production is associated with the activation of matrix metalloproteinase (MMPs), by myofibroblasts, which contributes to the mucosal remodelling and villous atrophy, typical of celiac disease (47).

Recently it has been identified one of the main immunogenic peptide of gliadin. This fragment of 33 aminoacids (33-mer), mapping N-teminal

region (57-68) of α-gliadin, is resistant to gastrointestinal degradation and is able to stimulate a strong immune response $CD4^+$ cells mediated in $DQ2^+$ celiacs (48).

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CHAPTER I

Identification of gliadin peptides restricted by the HLA Class I heterodimers encoded by A*0101 and B*0801 alleles, in strong linkage with the celiac disease-associated DR3-DQ2 haplotype

1.1 INTRODUCTION

1.1.1 Role of HLA Class-I restricted, adaptive CD8$^+$ T cells

Even though it has been demonstrated that CD is mediated by HLA class II genes, and that CD4$^+$T cells are responsible of intestinal damage, one of the most characteristic features of CD intestinal biopsies is a massive infiltration of the epithelium and lamina propria by CD8$^+$T lymphocytes. This CD8$^+$T cell infiltration is present in all form of celiac disease intestinal lesions, but very little is known about its function in celiac disease pathogenesis (1).

CD8$^+$T cells are responsible for the destruction of cells infected by viruses and other intracellular pathogens, and stimulate the immune-responses when immunogenic epitopes were presented on surface of Human Leukocyte Antigens (HLA) class I by Antigen Presenting Cells (APC).

HLA class I molecules are composed by two chains linked by non covalent bond: alpha and β_2-microglobulin. The alpha chain is highly polimorfic, while β_2-microglobulin results monomorfic. The alpha chain is formed by three extracellular domains, α1 (N-ter), α2, and α3 of about 90aa, by one transmembrane domain, and by one cytoplasmic domain. First two domains create a binding pocket able to interact with epitopes presented by APC. The third domain, fold in Ig-like (Immunoglobulin-like) domain. β_2-microglobulin, is composed by 100 aa, and plays an important structural role. The antigens were recognized and processed by APC, to be presented to T lymphocyte (2).

HLA class I are highly polimorfic, and this feature lead these molecules to recognize a high number of peptides. $CD8^+$T lymphocyte recognize peptides of 8-12 aa in length, when they are presented by HLA class I molecules (3). Following presentation cytotoxic T lymphocyte release INF-γ, and stimulate the lysis of target cells. These cells in fact own granules permeate with serine protease as well as granzyme-β (GrB), perforin and proteoglycan. $CD8^+$T lymphocyte interact with target cells and release for exocytosis the granzyme-β, and other enzyme responsible

of their lysis. Following $CD8^+$ cells, detached from these target cells, are able to recognize other HLA-peptides complex (4).

Several studies have suggested a potential role for class I-restricted T cells in autoimmune disease, as insulin dependent diabetes mellitus, or multiple sclerosis, but in celiac disease has not been demonstrated a clear genetic association with any HLA classes I genes.

Gianfrani and co-workers have reported a possible involvement of HLA class I genes in celiac disease, and examined the link between celiac disease and $CD8^+$T cell response. In particular, the Authors analyzed the immune responses to a panel of gliadin peptides high binders to HLA-A2 molecule, in A2 positive celiac patients. Thanks to this work has been described a 10-mer A gliadin peptides (pA_2 123-132) that stimulate HLA A2 restricted $CD8^+$ T lymphocyte from peripheral blood and intestinal mucosa of celiac patients (5).

By a further *ex vivo* study, it has been demonstrated that this peptide stimulates an immune response both in the lamina propria and in the epithelium in organ culture system. The cytotoxic $CD8^+$T cells activated in celiac mucosa of A2 celiac, react to epithelial $CaCo_2$ (HLA $A2^+$),

previously pulsed with pA_2 peptide, and stimulate the production of IFN-γ, and the release of Granzyme-B, and lead to the apoptosis of these target cells (6).

These evidences suggest that HLA class I could be play a role in the immunopathogenesis of disease.

Overall these data demonstrated that $CD8^+$T cells, which massively infiltrate the celiac disease intestinal mucosa, may also, in addition to a role in innate immunity (T cell receptor independent), already demonstrated perform specific types of response against gluten, following presentation of peptides by HLA class I, and participate to adaptive immunity.

To reinforce the hypothesis suggesting the possible involvement of adaptive $CT8^+$T cells in celiac disease, recently Davis and co-workers observed in peripheral blood of $DQ2^+$ celiac, following gluten challenge, along with the increase of gluten specific $CD4^+$T lymphocytes, a remarkable increased of αβ- and γδ- $CD8^+$T lymphocytes. These lymphocytes, expressed both gut homing markers such as CD103 (intestinal epithelial-homing markers αE), and $\beta 7$ integrins, and also the

activation marker CD30. In addition the Authors analyzed the T cell receptor (TCR) of T cell activated, and results suggest that $CD8^+$T cells are activated in antigen specific manner, in response to dietary gluten. Gluten oral administration induced a mobilization of $CD8^+$T lymphocytes from small intestinal mucosa to peripheral blood (7).

*1.1.2 Role of HLA class I A*01 and B*08 in celiac disease*

Celiac disease, as well as other autoimmune diseases, is due to genetic and environmental factors. For many autoimmune diseases the main genetic factors associated to the pathogenesis are the genes encoding the HLA complex. The genic area encoding for HLA complex, contain at least 128 genes, locate on chromosome 6, most of which (40%) are immune-genes. This genic complex is characterized by high gene density, and by strong linkage disequilibrium (8).

First celiac disease genetic studies, performed in the 70's, reported an association of disease with HLA Class I A*01 and B*08 genes (9, 10), only later studies associated the disease to HLA DQ2 and DQ8. HLA A1 and B8 molecules are found in strong linkage disequilibrium with HLA

Class II HLA-DR3-DQ2 that leads to the extended haplotype HLA A1-B8-DR3-DQ2. Aly and co-workers found that in this haplotype (spanning on a region of 2.9 Mb of MHC), HLA DQ2-DR3 are more conserved when associated to B8 than to A1 (11).

Since the early studies of genetic association, this extended haplotype has been found associated to several diseases including severe form of sclerosis (12). So, over the years it has been associated to high risk to develop multiple (auto)-immune diseases (Type1 diabetes (T1D), systemic lupus erythematosus, common variable immunodeficiency, HIV etc (13-17).

Despite that, genetic studies results about A1-B8-DR3-DQ2 and its involvement in celiac disease are long and complex, and often inconsistent (17-19). It's known that primary association of disease seems to be with HLA DQ2-DR3, but genetic susceptibility to celiac disease could be due to more than just HLA DQ2/DQ8 positivity, but also by the presence of other genes not identified yet, and HLA A1 or B8 could be one of these.

This haplotype was associated to different dysfunctions of immune system also in healthy controls. In particular, is responsible for an altered production of cytokines that lead to decrease of lymphocyte T helper 1 responses, and to the increase of T helper 2 responses (20-21). This unbalanced immune "responses" could be related to increase of risk to develop autoimmune disease by subjects carriers of this haplotype.

Despite that a clear and unique *in vitro* association of celiac disease to HLA class I A1 and B8 is not be demonstrated yet.

1.2 AIM

Aim of this project is to identify gliadin peptides able to activate a specific adaptive immune response restricted by HLA Class I A1 and B8. In particular we aimed to assess the frequency of $CD8^{+}$T cells specific for the identified HLA class I A1/B8- restricted epitopes in celiac patients. Also we investigated the frequency of extended haplotype A1-B8-DR3-DQ2 in our cohort of celiac volunteers.

The project proposed in this study comprises the following experimental phases:

- Typing of the HLA Class I A and B loci in celiac patients and non-celiac controls (for the selection of the study population).
- Typing of HLA Class II DR and DQ loci
- Identification of gliadin peptides that selectively bind to HLA Class I A*0101 or B*0801 molecules.

- Function assays to assess the immunogenicity of a panel of gliadin peptides selected on the base of their binding affinity to HLA A1/B8 in Celiacs.

- Further investigation of phenotype and function of T cells specific for immunogenic peptides by generating long term cytotoxic T cell lines.

1.3 MATERIALS AND METHODS

1.3.1 Study population and HLA class I and II typing

Population study was composed by 106 adult and young celiac patients (mean age 21.34- range 4-65), and by 35 healthy controls. All subjects were recruited in South Italy areas, the adult ones at "Moscati" Hospital of Avellino, while the young ones at the "Section of Pediatrics", Department of Translational Medical Science, of University of Naples "Federico II" (Table 1.3.1). All patients or their parents, in the case of children gave full informed consent to the experiments. Celiac patients received diagnosis of celiac disease in conformity to ESPGHAN (European Society for Pediatric Gastroenterology Hepatology and Nutrition) criteria. All celiac were on gluten-free diet (GFD) for at least 2 years, and were serum negative for anti-tTG and antiendomysium Ab (EMA).

From peripheral blood sample of participants were obtained peripheral blood mononuclear cells (PBMCs). T cells blast were generated following polyclonal stimulation of PBMCs with 0,5μg/ml phytohemagglutinin (PHA) (Roche, Basel, CH), then DNA were

extracted using commercial (Genomic DNA Miniprep kit, Sigma-Aldrich, St. Louis, MO) kits.

Pure genomic DNA was used to serologically typing for HLA class I A1 and B8 (HLA A and HLA B Low Res-AllSet Gold SSP), and class II (DRB1 and DQB1), using commercial Kit (AllSet^{+}SSP kit) (Life technologies, Carlsbad, CA). These kits are based on PCR method and use HLA specific oligonucleotide sequences as probes.

Twenty-four celiacs (mean age 23-range 5-45) within the HLA A1 or B8 positive population were enrolled for functional immunogenicity assays. Peripheral blood sample were collected to obtain PBMCs used in these assays.

In addition sixteen donor blood volunteers who result HLA A1^{+} and/or B8^{+} after either both flow cytometry (mAb Anti-HLA Class I B8, and A1-A36, Abcam, Cambridge, UK) and serological typing were selected. PBMCs derived from these subjects were used for functional assays.

Sigle	Age (ys)	Sex	HLA class I	HLA class II	Diagnosis	EMA
P1	28	F	A1+, B8-	DR7/DR5	celiac-GFD	Neg
P2	28	F	A1+, B8-	DR7/DR14	celiac-GFD	Neg
P3	41	F	A1+, B8+	DR3/DR1	celiac-GFD	Neg
P4	38	F	A1+, B8+	nd	celiac-GFD	Neg
P5	28	F	A1+, B8+	DR3/DR7	celiac-GFD	Neg
P6	40	F	A1+, B8+	DR3/DR5	celiac-GFD	Neg
P7	5	F	A1+, B8-	DR7/DR5	celiac-GFD	Neg
P8	18	F	A1+, B8-	DR3/DRX	celiac-GFD	Neg
P9	25	F	A1-, B8+	DR7/DR5	celiac-GFD	Neg
P10	45	F	A1-,B8+	DR3/DR5	celiac-GFD	Neg
P11	26	F	A1-,B8+	DR4/DR8	celiac-GFD	Neg
P12	30	F	A1-,B8+	DR3/DRX	celiac-GFD	Neg
P13	17	M	A1-,B8+	nd	celiac-GFD	Neg
P14	10	M	A1-,B8+	DR3/DRX	celiac-GFD	low pos
P15	11	F	A1-,B8+	DR3/DRX	celiac-GFD	Neg
P16	11	F	A1-,B8+	DR3/DRX	celiac-GFD	low pos
P17	8	M	A1-,B8+	DR3/DRX	celiac-GFD	Neg
P18	27	M	A1-,B8-	DR3/DRX	celiac-GFD	Neg
P19	42	F	A1-,B8-	DR3/DRX	celiac-GFD	Neg
P20	13	F	A1-,B8-	DR3/DR3	celiac-GFD	Neg
P21	16	M	A1-,B8-	DR3/DRX	celiac-GFD	Neg
P22	12	F	A1-,B8-	DR7/DR5	celiac-GFD	Neg
P23	15	M	A1-,B8-	nd	celiac-GFD	Neg
P24	20	M	A1-,B8-	nd	celiac-GFD	Neg

Table 1.3.1: Celiac patients enrolled in this study

1.3.2 Identification of candidate HLA A1 or HLA B8-resticted gliadin peptides

The entire amino-acid sequence of 2 α-, 2 γ- and 2 ω-gliadins (Gene-Bank) were analyzed using bioinformatic algorithms to identify the presence of HLA A*01 and HLA B*08 binding motif.

The binding motif of HLA A1 is S or T in position 2 and/or D and E in position 3, with Y at the C-terminus, while the binding motif of B8 is R or K in position 3 and/or 5, with L, I, V, M or F at the C-terminus (22).

This analysis has provided a certain number of gliadin peptides, predicted to have different binding affinity to the HLA A1 or B8 molecule. 97 of them showed high affinity (Binder threshold<IC50nM) and were selected for additional experiments.

These peptides were first synthesized and then purified using reverse-HPLC technique. Following synthesis, the 'purity' and 'identity' of peptides were confirmed using analytical reverse-HPLC and mass spectrometry.

1.3.3 Binding assay to purified HLA-A1 and B8 molecules

Next we confirmed the binding affinity for HLA A*0101 and for HLA B*0801 using an in vitro quantitative inhibition assay, based on purified HLA molecules and iodinated probe peptides. 1-10 nM of tested peptides iodinated were co-incubate 2hr at room temperature with purified HLA

A1 and HLA B8 molecules (obtained from EBV transformed cell lines Steinlein), in presence of 1 μM of β_2-microglobulin and protease inhibitor.

The unbound probe peptide fraction was separated from whole complex peptide/MHC class I by gel filtration and then quantified, to give a measure of the binding affinity.

The screening and synthesis of peptides were performed by the research group of Dr Alessandro Sette at La Jolla Institute for Allergy and Immunology, San Diego (CA).

1.3.4 EliSpot Assay

Peptides specific T cells response was observed using IFN-γ EliSpot Assay on freshly PBMCs. Gluten-specific T cell responses were observed using this assay on PBMCs, obtained by patients' peripheral blood samples.

PBMCs were isolated from heparinised whole blood using Ficoll (Ficoll-PaqueTM PLUS, GE Healthcare, UK) gradient separation. Cells ($2x10^5$)

were seeded in duplicate in complete medium [X-Vivo (Lonza, Basel, CH) enriched with 5% of heat inactivated Human Serum (Lonza, Basel, CH), 1% L-glutamine (2mM, Lonza, Basel, CH), and 1% Penicillin-Streptomicin (Lonza, Basel, CH)], on a 96 wells nitrocellulose backed plate (MAHA S4510- Millipore, Bedford, MA). Plates were pre-coated with anti (10μg/ml) IFN-γ mAb (Mabtech, Cincinnati, OH) over-night at 4°C in coating solution (NaHCO3 50mM/ Na2CO3 50mM).

Following cells incubation, plates were incubate with biotinylated anti-IFN-γ (Mabtech, Cincinnati, OH) for 2h at room temperature. Thereafter plates were washed with PBS-0,05% Tween20 (Sigma-Aldrich, San Louis, MO) and then were incubated with Streptoavidina HRP (BD-Pharmingen, Franklin Lakes, NJ) in a diluition buffer (PBS1X/BSA1%) (Sigma-Aldrich, San Louis, MO) for 1hr at room temperature. Following the addition of aminoethyl carbazole (AEC, Sigma-Aldrich, San Louis, MO) solution for five minutes, cytokines releasing cells can be detected as single spot using ELISPOT image analysed (A.EL.VIS, Hannover, Germany)

1.3.5 Generation of CTL lines from PBMCs

To generate CTLs, $4x10^6$ freshly PBMCs were plated in 1 ml of complete medium [(X-Vivo (Lonza, Basel, CH) enriched with 5% HS (Lonza, Basel,CH), 1% L-glutamine (2mM) (Lonza, Basel,CH), and 1% Penicillin-Streptomicin (Lonza, Basel, CH)] in presence of peptides (6μg/ml). At day 7 cells were re-stimulated with ($2-3x10^6$) autologous mononuclear cells (as described below), previously pulsed with gliadin peptides (6μg/ml). Interleukin 2 (R&D System, Minneapolis, MN) 40U/ml was added to feed cells every three days.

At 19-21 days after culture set-up, $CD8^+$ cells were separated from bulk culture using MACS immune-magnetic separation, according to manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany)

1.3.6 Generation of mononuclear cells from PBMCs & T cell Assay

To generate mononuclear cells at day 7 subsequent to generation of CTLs, autologous irradiated PBMCs were seeded at $2-3x10^6$ cells in 1ml in a 24 plate, and incubated for 2hrs at 37°C and 5% CO2. Non adherent

cells, were removed by washing twice with PRMI 2% HS [1% L-glutamine (2mM) (Lonza Basel, CH), and 1% Pen/Strep (Lonza, Basel, CH)]. Mononuclear cells were pulsed with peptides (6μg/ml/peptides) for 2hrs at 37°C and 5% CO2, in 1ml of complete medium enriched with 3ug/ml β_2-microglobulin (Calbiochem, San Diego, CA). After the incubation non adherent cells are removed from the plate, by twice washing, and CTLs previously collected are added to the mononuclear cell layer.

At 19-21 days after generation, CTLs and CD8 positive cells ($3x10^5$ cells) were assayed using IFN-γ EliSpot and/or ELISA to analyze immune response to gliadin peptides. Autologous PBMCs or HLA matched EBV ($1x10^5$/well) pulsed ON with gliadin peptides (10μg/ml), in presence of β_2-microglobulin (3μg/m, Calbiochem, San Diego, CA), were used as APC.

1.4 RESULTS

1.4.1 Identification of the study population

In order to investigate the role of HLA class I A*01 and B*08 alleles in celiac disease, and to identify gliadin epitopes restricted by HLA class I A1/B8 molecules, we screened volunteers with celiac diseases or healthy donor. To assess the frequency of HLA Class I A*01 and B*08 alleles our selects subjects, carrying the HLA Class I A*01 and/or B*08 alleles, were submitted to a complete HLA Class I and II genotyping.

Results of HLA class I typing have shown that overall 42,4% celiacs results HLA A1 and/or B8 positive. In particular patients $A1^{+}/B8^{-}$ were 18,9%, while patients $A1^{-}/B8^{+}$ were 9,4% and $A1^{+}/B8^{+}$ patients were 14,1%.

After HLA class II typing we found that frequency of DR3-DQ2 is 42%. Among our $DR3^{+}$ the 65,4% of subject were A1 and/or B8 positive, in particular the $A1^{+}/B8^{-}$ subject are the 7,69%, the $A1^{-}/B8^{+}$ subject are the 26,92%, while the $A1^{+}/B8^{+}$ are 30,77%.

These results seem to be in accordance with previously published data, that showed a preferential association of HLA class I A1 and B8 to HLA class II DR3 (13).

1.4.2 Identification of HLA-I A1 or B8 restricted gliadin peptides

Following the identification of a population study, we have attempted to identify gliadin toxic epitopes, potentially recognized by HLA class I A1 and/or B8, using the already known binding motifs to HLA Class I A1or to B8, molecules (22).

The screening of the entire aminoacid sequence of 2 α-, 2 γ- and 2 ω-gliadins, provided hundreds of peptides, candidate epitopes A1/B8 restricted. We have chosen 97 peptides of 9-10 aminoacid lenght with the highest score of binding (data not shown).

Predicted peptides mapped in all gliadins examined (alpha-, gamma- and omega-gliadin). 24/97 (24,7%) were found in the omega gliadins, 25/97 (25,8%) in the alpha gliadins and 48/97 (49,5%) in the gamma gliadins.

Of all peptides identified 47 (48,45%) are potential HLA A1 binder, 47 (48,45%) HLA B8 binder, and 3 (3%) were predicted bind both HLA A1 and B8 molecules.

All peptides were synthesized, and following quantitative inhibition assay, it has been demonstrated that only 23 peptides 23,7% (23/97) had a significant thresholds binding affinity (IC50<500nM) to purified HLA class I molecules (Table 1.4.1).

Peptide	Protein	Position	Sequence	Selection basis	Binder
# 1	α-gliadin	158	SSQVLQQSTY	**A*0101**	**Yes**
# 2	ω-gliadin	414	SEEPSPYQQY	**A*0101**	**Yes**
# 3	α-gliadin	217	VSFQQPQQQY	**A*0101**	**Yes**
# 4	ω-gliadin	12	MAMKIATAA	**B*0801**	**Yes**
# 5	γ-gliadin	159	FLLQQCKPV	**B*0801**	**Yes**
# 6	γ-gliadin	160	LLQQCKPVSL	**B*0801**	**Yes**
# 7	γ-gliadin	188	LLQQSKPASL	**B*0801**	**Yes**
# 8	γ-gliadin	189	LQQSKPASL	**B*0801**	**Yes**
# 9	α-gliadin	254	FEEIRNLAL	**B*0801**	**Yes**
# 10	ω-gliadin	11	AMAMKIATA	**B*0801**	**Yes**
# 11	γ-gliadin	161	LQQCKPVSL	**B*0801**	**Yes**
# 12	γ-gliadin	224	QGMHILLPL	**B*0801**	**Yes**
# 13	ω-gliadin	22	LLSPRGKEL	**B*0801**	**Yes**
# 14	γ-gliadin	165	FPQQQRPFI	**B*0801**	**Yes**
# 15	ω-gliadin	10	LAMAMKIATA	**B*0801**	**Yes**
# 16	γ-gliadin	213	VMRQQCCQQL	**B*0801**	**Yes**
# 17	α/β-gliadin	1	MKTFLILAL	**B*0801**	**Yes**
# 18	α-gliadin	141	LIPCRDVVL	**B*0801**	**Yes**
# 19	γ-gliadin	31	WLQQQLVPQL	**B*0801**	**Yes**
# 20	γ-gliadin	287	LEAIRSLVL	**B*0801**	**Yes**
# 21	γ-gliadin	286	QLEAIRSLV	**B*0801**	**Yes**
# 22	ω-gliadin	14	MNIASASRL	**B*0801**	**Yes**
# 23	γ-gliadin	203	CAAIHTIIH	**B*0801**	**Yes**

Table 1.4.1: List of gliadin peptides with the highest score to bind HLA class I $A1^{+}$ or B8 molecules

In particular 3/97 (3,1%) peptides have shown significant affinity to HLA A1 purified molecules, while 20/97 (20,62%) to purified HLA B8 molecules.

These 23 peptides have been used for additional experiments.

*1.4.3 Immune response to HLA A*01 and B*08 restricted peptides in peripheral blood cells*

Following identification and synthesis, we assayed the immunogenic properties of selected peptides. In particular, we assessed the ability of PBMCs derived from celiac and healthy volunteers HLA A1 and/or B8 positive and celiac A1/B8 negative to recognize peptides, using IFN-γ EliSpot Assay.

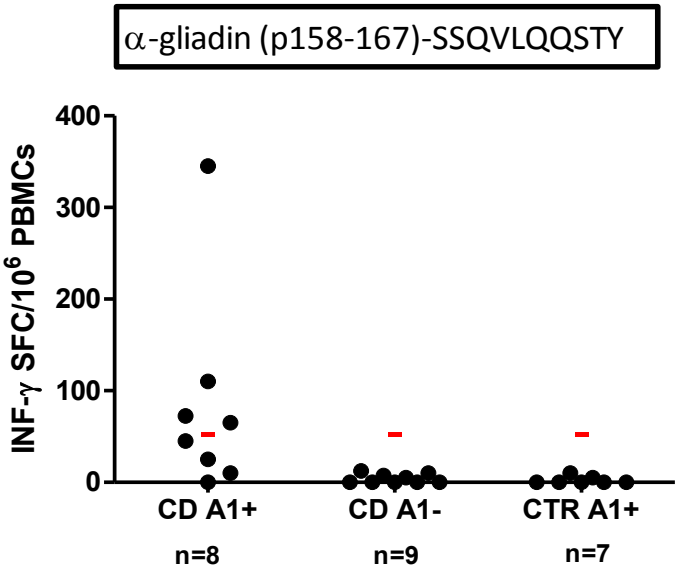
Each of the 3 A1 binder peptides were assayed in celiac (both HLA A1 positive and HLA A1 negative), and A1 positive healthy controls.

Results were shown as Net IFN-γ-SFC/10^6 cells. The means plus 2SD of this value for each A1 peptides were calculated in HLA A1 negative celiac patients. The responses of each patient were considered positive

when exceeding these values. We considered the positive response significant, when it was detected in at least 3 patients.

As shown in figure 1.4.1 $p_{158-167}$ derived from ω-gliadin stimulated the activation of INF-γ secreting cells in 4/8 celiac $A1^{+}$, corresponding to 50% of responders' population.

Also we found a significantly IFN-γ production in response to $p_{414-423}$ of ω-gliadin in three/8 patients corresponding to 37,5% of population.



A)

B)

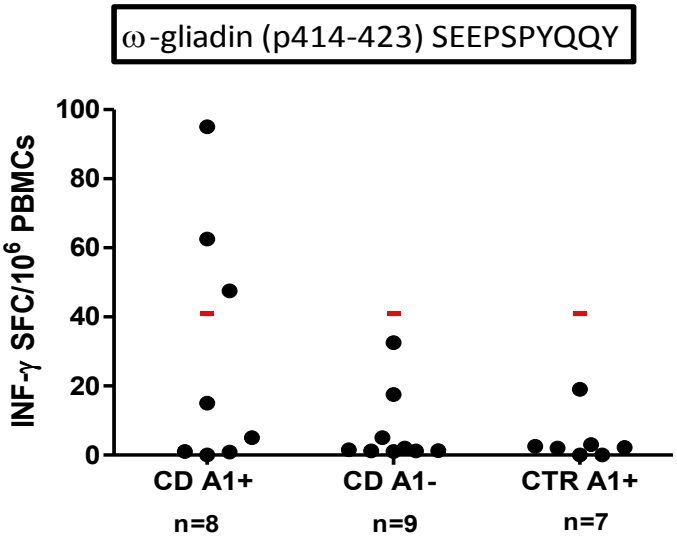


Figure 1.4.1: Gliadin-specific IFN-γ-releasing cells detected in PBMCs of HLA-A*01 positive and negative celiac patients and healthy controls in response to HLA class I A1 peptide p#1(A) and p#2 (B). Red lines indicate the mean plus 2 standard deviations of responses from $A1^-$ negative celiac subjects.

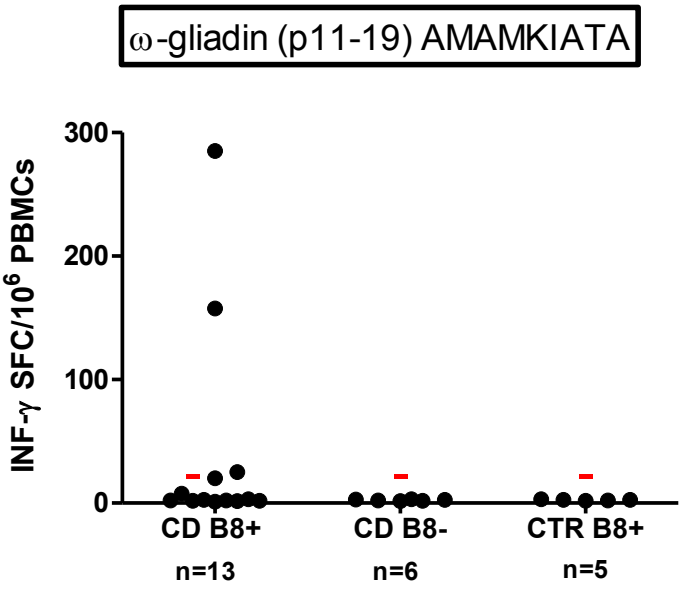
In no cases a significant response was observed in healthy controls.

All these data demonstrated that 66,65% of HLA A1 restricted peptides resulted able to activate an immunogenic response in PBMCs in at least 3/eight celiac.

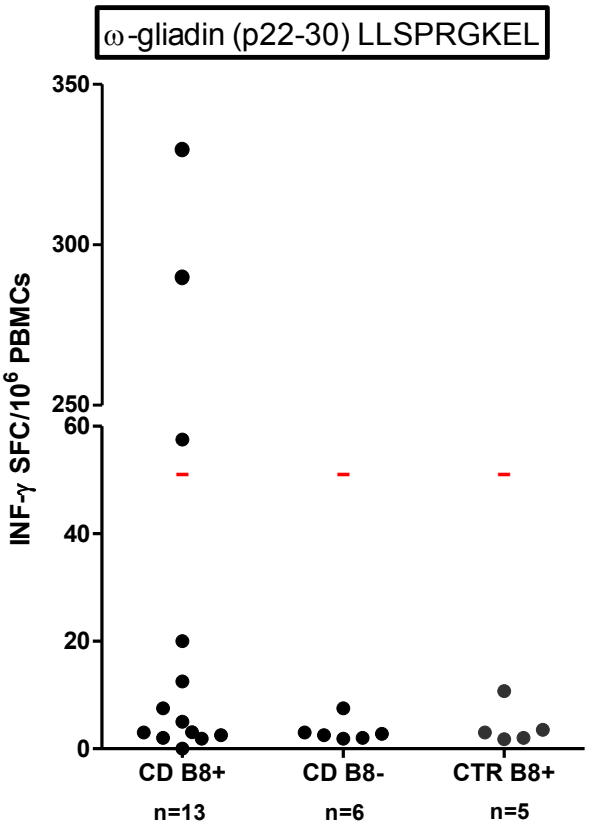
Same responsiveness criteria were used to select positive HLA B8 restricted peptides.

PBMCs of both celiac and healthy controls cells were assessed towards twenty HLA B8 binder epitopes (Fig.1.4.2).

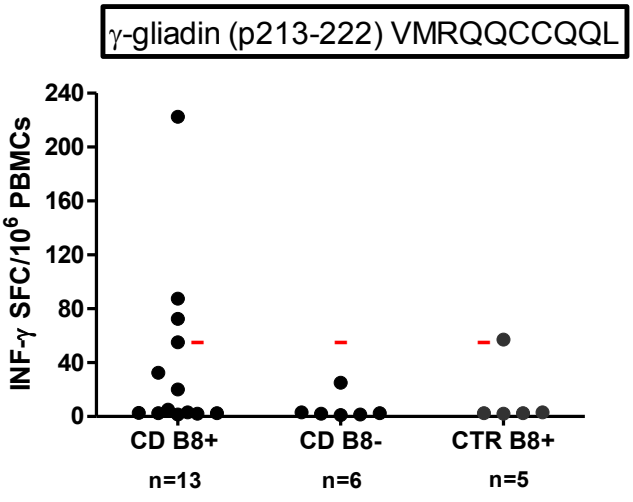
Results have shown that seven HLA B8 peptides (p#10, p#12, p#13, p#16, p#17, p#20, p#23) stimulate PBMCs, in at least three B8 positive celiac, corresponding to 23,07% of population to produce IFN-γ. In no cases we found a positive response to these peptides by healthy controls.



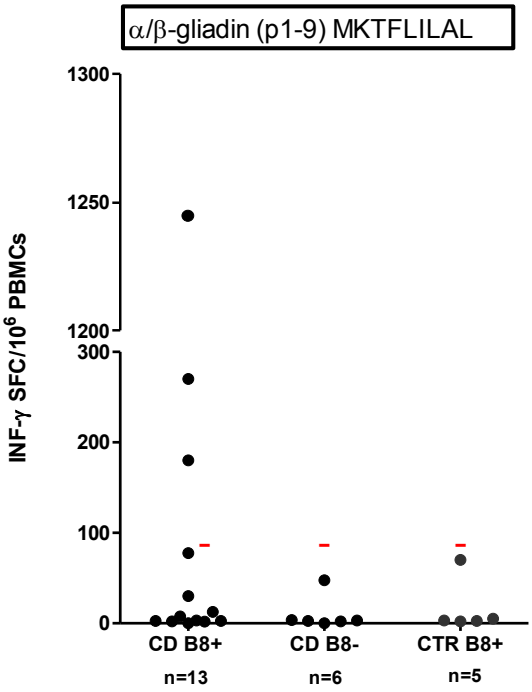
A)


ω-gliadin (p22-30) LLSPRGKEL
INF-γ SFC/10^6 PBMCs
350
300
250
60
40
20
0
CD B8+
n=13
CD B8-
n=6
CTR B8+
n=5

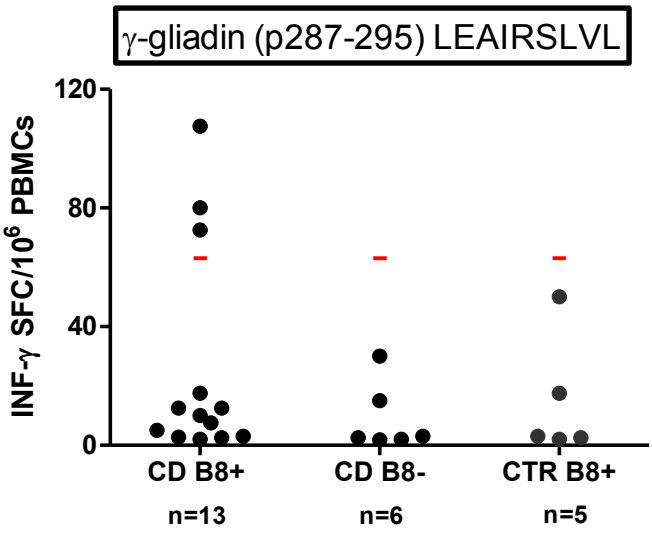
B)


γ-gliadin (p213-222) VMRQQCCQQL
INF-γ SFC/10^6 PBMCs
240
200
160
120
80
40
0
CD B8+
n=13
CD B8-
n=6
CTR B8+
n=5

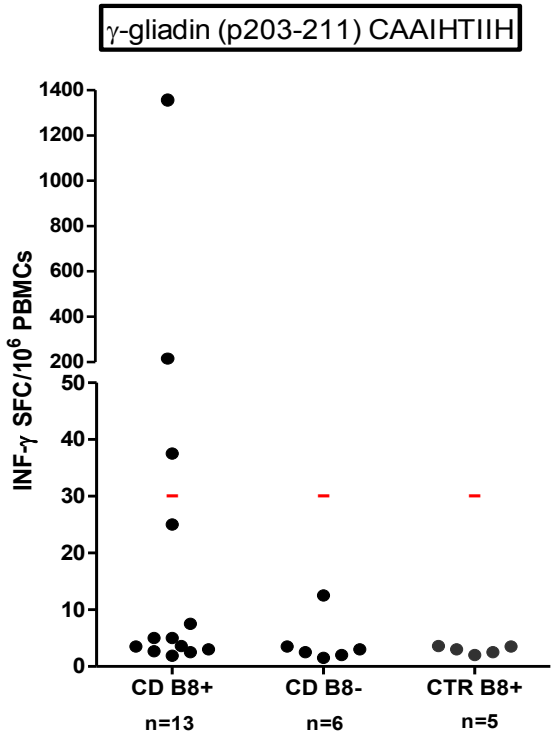
C)


α/β-gliadin (p1-9) MKTFLILAL
INF-γ SFC/10^6 PBMCs
1300
1250
1200
300
200
100
0
CD B8+
n=13
CD B8-
n=6
CTR B8+
n=5

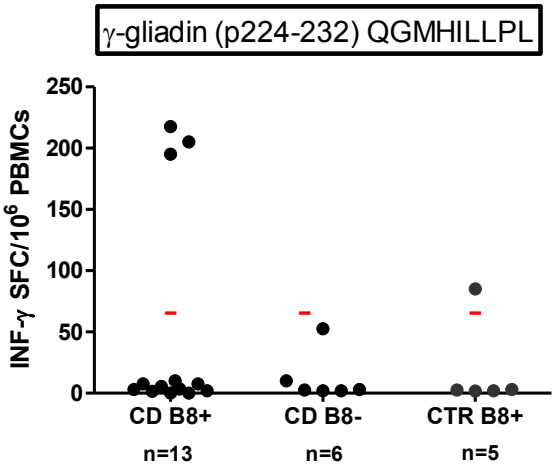
D)


γ-gliadin (p287-295) LEAIRSLVL
INF-γ SFC/10^6 PBMCs
120
80
40
0
CD B8+
n=13
CD B8-
n=6
CTR B8+
n=5

E)



F)



G)

Figure 1.4.2: Gliadin-specific IFN-γ-releasing cells in response to HLA class I B8 peptide [p#10(A), p#12(B), p#13(C), p#16(D), p#17(E), p#20(F), p#23(G)]. Results are shown as Net IFN-γ-SFC/10^6 cells. Red lines indicate the mean plus 2 standard deviations of responses from $B8^-$ negative celiac subjects

Peptide $p_{286-294}$, stimulated responses in eight/13 celiac corresponding to 61,54% of population. Responses of healthy control HLA B8 were not significant (fig1.4.3).

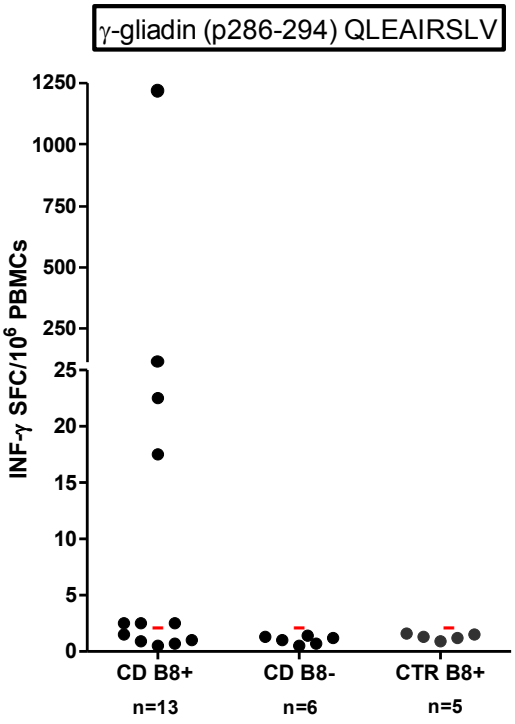


Figure 1.4.3: Gliadin-specific IFN-γ-releasing cells in response to HLA class I B8 peptide (#21). Results are shown as Net IFN-γ-SFC/10^6 cells. Dash lines indicate the mean plus 2 standard deviations of responses from $B8^-$ negative celiac subjects

These data indicated that 34,8% of restricted B8 peptides stimulated an immune response in PBMCs of B8 celiac.

25% (2/8) of all immunogenic peptides derived from α-gliadin, 12,5% (1/8) derived from α/β-gliadin, while the rest (62,5%-5/8) derived from γ-gliadin.

Overall these data demonstrated that different gliadins have immunogenic peptides able to recall a secondary immune-response in PBMCs of celiac with HLA class I A1 or B8.

*1.4.4 Immune response to HLA A*01 and B*08 restricted peptides in fresh peripheral blood cells*

To further analyze the response obtained, we summarized in table 1.4.2 the profile of peptides recognition for each A1 patients. Of all A1 positive selected patients five/8 were responders. Patients P5 and P8 were found reactive to both A1 immunogenic epitopes, while P1, P6 and P7 recognized only one/2 immunogenic peptides. Three celiac were not reactive to any peptides. The 60% of responders results $DR3^{+}$.

code	gliadin active peptides		RESPONDER PATIENTS								
	A1 BINDERS PEPTIDES		P1	P2	P3	P4	P5	P6	P7	P8	
#1	α-gliadin	SSQVLQQSTY	■				■	■		■	4/8
#2	ω-gliadin	SEEPSPYQQY					■		■	■	3/8

Table 1.4.2: Profile of recognition of HLA A1^{+} restricted peptides in celiac patients

In table 1.4.3 is shown the profile of responses to HLA B8 peptides by celiac B8 positive. As for A1 responders, the responses to B8 epitopes were heterogeneous among 9/13 responders, despite two patients gave a significant response to almost all immunogenic peptides. About 67% of responders were DR3^{+}.

	gliadin active peptides		RESPONDER PATIENTS													
	B8 BINDERS PEPTIDES		P3	P4	P5	P6	P9	P10	P11	P12	P13	P14	P15	P16	P17	
#10	ω-gliadin	AMAMKIATA								■	■			■		3/13
#12	γ-gliadin	QGMHILLPL							■		■			■		3/13
#13	ω-gliadin	LLSPRGKEL				■					■			■		3/13
# 16	γ-gliadin	VMRQQCCQQL			■						■			■		3/13
#17	α/β-gliadin	MKTFLILAL							■		■			■		3/13
#20	γ-gliadin	LEAIRSLVL			■				■	■	■			■		3/13
#21	γ-gliadin	QLEAIRSLV	■			■			■	■	■		■	■		3/13
#23	γ-gliadin	CAAIHTIIH			■						■			■		3/13

Table 1.4.3: Profile of recognition of HLA B8^{+} restricted peptides in celiac patients

*1.4.5 Immune responses to HLA A*01 and B*08 restricted peptides in CTLs*

Next we investigated the nature of responses obtained. In particular we generated cytotoxic T cells stimulating in vitro PBMCs, from two B8

positive patients, with a pool of immunogenic B8 epitopes, whose response results positive by previously functional assays. Next we evaluated IFN-γ secreting cells in response to mentioned above peptides, and also performed the assays on bulk culture, and on purified $CD8^+$ cells, obtained following immunomagnetic CD8 separation.

In figure 1.4.5 is shown the representative response obtained in patient 3 in response to gliadin, and to $p_{224-232}$ peptide and to p_{11-19} peptide. Results were shown as Net IFN-γ secreting cells.

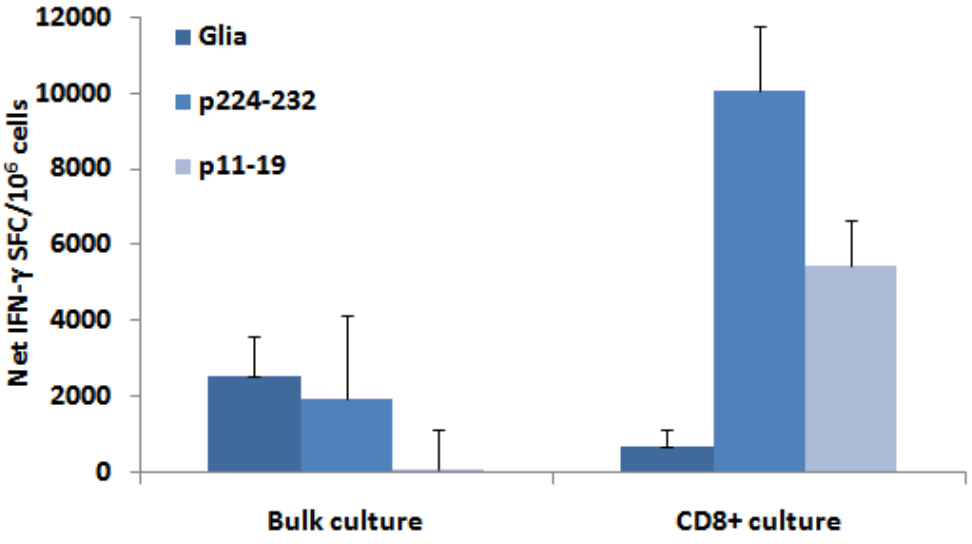


Figure 1.4.5: Net IFN-γ/SFC 10^6 obtained on bulk culture, and on $CD8^+$ T cells purified with magnetic beads in response to gliadin and to p11-19 and p224-232 peptide.

Results have shown that in bulk culture net IFN-γ secreting cells were in response to p_{11-19} in the range 166,5-110/10^6 (mean 133/10^6±47), in response to $p_{224-232}$ in the range 3496-399/10^6 (mean1948/10^6 ±2189). Following immunomagnetic separation we found net-IFN-γ secreting cells were in response to p_{11-19} in the range 6327-4628/10^6 (mean 5477,85/10^6 ±1200), in response to $p_{224-232}$ in the range 11255,4-8891/10^6 (mean 10073,25±1671).

In addition we performed a phenotypic analysis of these two cell populations. In one of this case in bulk culture 51,91% of population is composed by $CD3^+CD8^+$ cells, and this population contains ~0,01% (133,25/10^6) p_{11-19} responsive cells, and ~0,2% (1948/10^6) $p_{224-232}$ responsive cells Following immunomagnetic separation we found that $CD3^+CD8^+$ became 93,92%, and the percentage of p_{11-19} responsive cells increase up to ~0,55%, while $p_{224-232}$ responsive cells became ~1% .

Phenotypic analysis confirmed that these immune-responses were mediated by $CD8^+$ cells.

Overall these data indicated that in peripheral blood of celiac, specific CTLs produced IFN-γ in response to peptides restricted by HLA class I.

1.5 DISCUSSION

Celiac disease is an auto-immune disorder in which both innate and adaptive immunity are involved. It has been widely characterized the role of $CD4^+$T lymphocytes in the mucosa damage, and the involvement of HLA class II DQ2/DQ8 in the disease (23). In fact, it has been recently identified the complete repertoire of the immunogenic gluten peptides and the immune-dominant epitopes, eliciting a $CD4^+$T cells response (24).

There is a general consensus on the key role of $CT8^+$T lymphocytes, massively infiltrating intestinal mucosa of all celiac disease forms, in the villous atrophy. In particular, this prominent pathogenic function was mainly attributed to the intraepithelial $CD8^+$T lymphocytes of the innate immunity branch (25). These $CD8^+$ IELs would be armed to kill enterocytes after gluten exposure, through a mechanism TCR-independent. Despite that, very little is known about the role of adaptive T $CD8^+$ lymphocytes, and the possible involvement of HLA Class I molecules in CD pathogenesis is not completely clarified.

First evidences have demonstrated that gliadin contains a peptide able to activate adaptive $CD8^+$T lymphocytes exclusively in celiac disease patients. This peptide mapping the 123-132 sequence of α-gliadins is restricted by the HLA Class I A2 molecule and is specifically recognized by HLA-A2 positive CD subjects. However, there is no strict genetic association with HLA A*0201 gene, since it is carried by almost 30-40% of celiac subjects, a percentage non different from that of the general Caucasian population (26).

In the present study, we have expanded the analysis to A*01 and B*08, two other HLA Class I alleles usually present in Caucasian population. We reported that several gliadin peptides with high binding affinity to HLA class I A1 and B8 molecules are able to stimulate a $CD8^+$T cell response in celiac patients A1/B8 positive.

In order to explore the role of HLA class I restricted $CD8^+$ T cells in CD, we have screened the entire sequences of two α- two γ- and two ω-gliadins, for the presence of peptides predicted to bind A1 and B8 molecules using bioinformatic algorithms available from the IEDB (www.iedb.org) based on A1 and B8 binding motifs.

Remarkably, our results demonstrated that all families of gliadin (α-, γ-, and ω-gliadins) contain hundreds of peptides potential binder to A1 and B8. The top 1% scoring 9- and 10-mer peptides were synthesized and tested in MHC-peptide binding assays using purified A*0101 and B*0801 molecules. Overall, 23 peptides had an high *in vitro* affinity, and in particular, three peptides bound HLA A1, while the other twenty bound HLA B8 and were selected for immunogenic assays.

We demonstrated that ten gliadin epitopes restricted by HLA class I A1 (two peptides) and B8 (eight peptides) recall a secondary immune response in PBMCs of celiac patients under treatment. Further, for two peptides ($p_{11\text{-}19}$ and $p_{224\text{-}232}$) the cells involved in the induction of response were $CD8^{+}$T lymphocyte.

In both conditions the activation of immunonogenic pattern was evaluated measuring IFN-γ secreting cells. IFN-γ represent one of most pro-inflammatory cytokines involved in the intestinal damage (27).

In addition genotyping results confirmed in our Campania population the strong association of HLA class I A1 and B8 to HLA class II DR3.

Interestingly, functional assays revealed a very heterogeneous recognition pathway among CD volunteers, since we have not found a prevalent response towards a specific peptide(s). By contrast, we observed that each of 23 different peptides were recognized by at least one CD patients (data not shown). To avoid any bias, our cut-off was very selective. We calculated means plus 2SD of Net-γ SFC/10^6 cells obtained in celiac negative for HLA class I A1 and B8. The responses to each peptide were considered positive when exceeding these values. We considered immunogenic those peptides that gave a positive response in almost 3 of tested patients.

On the base of our criteria of positive response, we verified that two of HLA class I A1 restricted peptides ($p_{158-167}$ and $p_{414-423}$) corresponding to 66,65%, and eight of HLA class I B8 restricted peptides ($p_{11-19,}$ $p_{224-232}$, p_{22-30}, $p_{213-222}$, p_{1-9}, $p_{287-295}$, $p_{286-294}$ and $p_{203-211}$), corresponding to 34,8%, can recall a T cell responses in peripheral blood cells of celiacs respectively A1 and B8 positive, and can be considered immunogenic. However peptide $p_{158-167}$ derived from α-gliadin and restricted by HLA A1, and peptide $p_{286-294}$ derived from γ-gliadin, and restricted by HLA

B8, results more immunogenic than other. $p_{158-167}$ activated a T cell response in 50% (4/8) of $A1^+$ celiac, while $p_{158-167}$ in 61,54% (8/14) of HLA $B8^+$ patients. We hypothesized that these two peptides could be presented by HLA class I molecules more easily and strongly than other, and they have more immunogenic sequences than other peptides.

Also, in our T cell responses we have not found any significant response by PBMCs of healthy controls, enrolled on the base of their positivity for A*01 or B*08 alleles, to any candidate gliadin peptides. One exception was represented by one healthy control whose response to $p_{224-232}$, exceeded the cut-off values. We hypothesized that this subject (HLA $A1^-$ $B8^+$) could be a case of undiagnosed celiac disease, or the activation of immune-response could be a consequences of cross-reactivity to this peptide by different HLA Class I (for example by A36, B35 and/or B62).

Many studies concerning prediction of binding, have asserted that binding affinity correlates with immunogenicity (28-30). Sette and coworkers have shown that 50% of all high-affinity binders identified in an HLA A*02 mouse systems are immunogenic (31). In our study we found, in accordance with these data that among 23 epitopes selected for

functional assays, based on high binding affinity to HLA class I molecules, ~43% results immunogenic. These data confirmed that binding analysis provided a great advantage in identification of peptides potentially epitopes of T cells.

By itself these results were important because they show for the first time that gliadins have sequences potentially binders to HLA A1 or B8. Importantly, genes coding for A1 and B8 heterodimers are part of an extended haplotype represented by HLA A1-B8-DR3-DQ2 that is associated with several autoimmune disease (such as Systemic lupus erythematousus, diabetes mellitus, ecc) (13).

Results of screening showed that γ-gliadins contains the highest number of peptides binding to HLA class I than the other two gliadins (approximately 49,5% of all predicted peptides, compared to % 25,7% of alpha and 24,7% of omega). In addition, results obtained with *in vitro* binding assays (52,17% were gamma-gliadins compared to 21,7% of alpha, and 26% of omega), and with functional assays (50% of all A1 and B8 immunogenic peptides derived from gamma-gliadins compared to 37,5% from omega, and 25% from alpha gliadin), confirmed that

HLA class I preferentially presented γ-gliadin peptides. These data could indicate that γ-gliadin play an important role in the immune-pathogenesis of celiac disease, in accordance to previous studies indicating the high immunogenic properties of some γ-gliadin peptides on $CD4^{+}$T cells (32).

In order to exclude that the activation of immune response was the results of presentation of gliadin peptides by HLA DQ2 molecules, we screened the sequences of all peptides for the presence of consensus sequence for binding to DQ2/DQ8. It's known that binding motif sites to HLA class I A1 and B8 are very different to binding motif sites of HLA class II DQ2/DQ8 (with a E in position 4, 6, 7 for DQ2 and E in position 1 and 9 for DQ28). We found that among all A1 and B8 binders peptides analysed in our study, only two had classical QXP site (FPQQQRPFI restricted by HLA B8, and VSFQQPQQQY restricted by HLA A1) and could determinate a cross-reactivity response. However, we have not found an *in-vitro* significant immunological response by PBMCs to both this peptides, thus excluding any immune activity in our celiac patient cohort.

Next, we further investigated the phenotype of PBMCs reacting to immunogenic peptides by generating short-term cytotoxic $CD8^+$T cell lines, raised toward the most active epitopes. Our results have shown that two peptides $p_{11\text{-}19}$ and $p_{224\text{-}232}$ were able to stimulate specific CTLs to produce IFN-γ. Phenotypic analysis confirmed that $CD3^+CD8^+$ cells mediated these responses, as established by ~41 fold increase in IFN-γ levels obtained in purified CD8 cells in response to $p_{11\text{-}19}$, and by ~5,2 fold increase obtained in response to $p_{224\text{-}232}$.

The low frequency of specific cells (from <0,001% to 0,1%), could be explained by the fact that we have analysed response in peripheral blood of subjects on gluten free diets. It is most likely that the frequency of these cells could be much higher after a short gluten challenge. In concordance with this hypothesis, Han and co-workers (55) found an increase of $CD8\alpha^+\beta^+$ cells from <0,1% in basal condition (day0) to 1,2% (day6) following gluten challenge along with an increase of $\gamma^+\delta^+$ cells (from <0,1% at day 0, to 6,8% at day 6).

Overall these data shown that gliadin peptides were able to stimulate PBMCs or cytotoxic cells to produce IFN-γ, and for this reason could

contribute to activation or maintenance of immune-response and so contribute to intestinal damage.

Interesting it's widely demonstrated by several studies the involvement of cytotoxic cells in the pathogenesis of different autoimmune disease.

Extensive evidences have demonstrated the role of cytotoxic $CD8^+$T cells and HLA class I in diabetes. Many studies have shown these evidences in mouse model. It has been clarified that Nonobese mouse model (NOD) null for expression of β_2-microglobulin lack the ability to develop insulitis and diabetes in NOD mouse (33, 34). Unlike when β_2-microglobulin was deleted only from β-cells the progression of disease was decrease (35).

Cytotoxic cells were also involved in human T1D and in islet allograft rejection. Islet infiltrating pancreatic cells was predominantly $CD8^+$ (36). These evidences were reinforced recently when $CD8^+$ cells were evidenced in the islet of organ donors who are positive for islet cell auto-antibodies (37).

Further *in vitro* evidences have established that human β-cells were destructed by CTL in MHC specific manner (38).

Coppieters et al, has shown first evidences of presence of islet-reactive CD8 T cells in insulitic lesions from patients with recent-onset and also longstanding T1D, confirming their role of these cells in progression of disease (39).

In contrast several auto antigens epitopes recognized by $CD8^{+}T$ in diabetes, were identified in proinsulin, (40, 41), glutamic acid decarboxilase (GAD) (42) and glucose 6 phospatase catalytic subunit releted protein IGPRP.

Autoreactive CD8 T cells play a role moreover in Multiple Sclerosis (MS) since expansions of CD8 T cells were found in the blood, and in the cerebrospinal fluid of MS patients (43), suggesting that myelin antigens can indeed be recognized by human MHC class I-restricted CD8 T cells.

Our results were in accordance with overall these evidences, these data boost to carry on in the investigation of $CD8^{+}T$ cells role in celiac damage, since only recently has been posed attention on involvement of this bunch of adaptive immunity.

Results of genotyping have shown that among our celiac population the frequency of HLA A1 or B8 were 42,5%, but interesting, among our

DR3^{+} the percentage increase to 65,4%. Experimental results have shown that among all celiac responders the 58,8% results to be DR3^{+}, while the rest were DR5/DR7, with only one exception represented by one celiac DR4/DR8. All these data underlay the linkage of A1 and B8 to DR3. Our findings were consistent with previously published data that demonstrated the involvement of this haplotype in the immune mediated response in patients with celiac disease (13).

In this project we decided to identify gliadin epitopes potentially recognized by CD8^{+} cells. At the presents only few previously studies demonstrated the involvement of T CD8^{+} lymphocyte TCR dependent response in celiac disease. In particular as mentioned already Gianfrani and co-workers demonstrated that, in celiac patients with HLA class I A2 positive celiac, the peptide 123-132 of A-gliadin, induced a T CD8^{+} cell response responsible of tissue damage.

It known that first celiac disease genetic association studies reported an association of disease with HLA Class I A*01 and B*08 genes (9, 10). HLA A*01 and B*08 are often related in the auto-immune haplotype HLA A1-B8-DR3-DQ2. This haplotype is highly conserved, and, as

widely demonstrated, was found associated to high risk to develop multiple autoimmune disease (13).

This genic complex, including HLA A1-B8-DR3-DQ2, is characterized by high gene density and by strong linkage disequilibrium (11) responsible for the transmission of the extended haplotype.

As mentioned above, the genetic study results, about this haplotype, are often inconsistent. A previously study showed in Spanish children population that among HLA A-B-C, the A1-B8-Cw7 haplotype (A19-B12-Cw7/A1-B8-Cw7), and among HLA A-B-DR, A1-B8-DR3 haplotype results most frequent in celiac than healthy controls (17).

In addition the haplotype B8-DR3-DQ2 was found over-expressed (44) in patients with atypical form of celiac disease than the other. In patients with both, celiac disease and type 1 diabetes, has been demonstrated that this haplotype is more frequent in celiac, and, B8 is main risk factor to develop celiac disease presents in this haplotype (18). B8, in fact, contributes to increase of approximately four fold the CD development risk given by DR3-DQ2 (fundamental to CD development) both in Italian and Finnish (18).

HLA B8 probably is crucial for antigen basic for development of disease, as confirmed by demonstration of major association of this HLA to CD patients than diabetes. B8 is major risk factor in the haplotype and contribute to develop of disease more than other HLA class II genes.

We identified more immunogenic peptides binder to HLA B8, than A1. These features could be related to higher risks to develop disease associated to HLA B8 than A1.

For all this reason in this project we decided to evaluate the role of this extended haplotype, looking at the genetic penetrance of HLA A*01 and/or B*08 alleles in our population study. This might be useful to help to understand the genes involved, and better characterize the genetic risk of celiac disease, and the immune mediated mechanisms responsible for the development of disease.

In future will be important to further characterize the role of $CD8^{+}$T cell mediated responses in this disease, for example to evaluate if these cells produce IL-21 and IL-17, two cytokines modulated (45) in celiac disease, and to investigate whether HLA A1/B8 restricted $CD8^{+}$ T lymphocytes contribute to the lesion of intestinal mucosa of CD patients.

Through this research project we aim to better characterize the involvement of the adaptive lymphocyte response mediated by $CD8^+$T cells in celiac disease. Only if we identify all immune mechanism of disease, we can image and design a new therapy.

The characterization of toxic fractions of gliadin, capable of generating a lymphocyte response in patients with HLA class I A1 and/or B8, would allow a better understanding on the molecular mechanisms responsible for the development of small intestinal lesions.

This study could be useful to confirm the role of genetic risk of the extended haplotype DR3-DQ2-A1-B8 in developing celiac disease. This information could be important to allow risk stratification in family with first degree relative with the celiac disease.

This project could provide new insights into the genetic and immunological components responsible for the development of celiac disease, which at the time, have not been fully clarified yet.

Given the strong linkage disequilibrium of A*01 and B*08 with the DR3-DQ2 haplotype, and the immune-stimulatory properties of A1 and

B8 gliadin peptides, the potential role of A1 and B8 gene/molecules in CD pathogenesis should be revaluated.

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CHAPTER 2

Repertoire of epitopes recognized by CD4 T cells in children with early or overt celiac disease.

2.1 INTRODUCTION

2.1.1. Is celiac disease different among adults and children?

It has been reported that clinical manifestations are different among adult and children with CD. Vivas and co-workers have suggested an age related pattern in clinical presentation of CD, since the disease presents with more pronounced symptoms, and with a severe villous atrophy in young children (1). Younger children, also, present with abdominal complaints, bowel movement disorder, and pronounced GI symptoms than older children with CD (2). By contrast, both atypical and silent forms present similar clinical manifestations among the different age related groups (2), with exception of anemia that is prevalent in adult, whilst the short stature is more prevalent in children and dermatitis herpetiformis affects mostly adults (3).

Furthermore, children with CD show a faster resolution of the inflammatory and intestinal damage upon gluten free diet, and have a

reduced risk to develop complications, such as autoimmune disease in comparison to adults (4). In fact, the association of CD with auto-immune disease varies in age related manner, and it has been found a positive correlation of auto-immune diseases incidence with the age of CD diagnosis (5-7). This may be related to duration of gluten exposure until diagnosis (7). One of the key questions is whether the differences in clinical phenotype between children and adult celiac subjects depend on differences in the immunological response to gluten.

Since the time elapsed from the development of disease to clinical diagnosis may vary in the order of weeks, months up to many years, it is not possible to characterize the primary immune response to gluten, particularly the repertoire of gluten peptides inducing the primary T cell activation in CD mucosa. However it is possible to investigate the secondary memory T cell responses generated in celiacs at the different disease stages (8, 9). Several experimental approaches including long-term T cell lines (TCLs) and T cell clones (TCCs) raised from small intestinal mucosa or peripheral blood responses after gluten oral challenge have been used to identify the stimulatory gluten sequences

(10). It has been demonstrated that the great majority of CD4 T cell-mediated immune responses from adult celiac patients were directed against gluten peptides when deamidated by tissue transglutaminase (TG) (11). Since the activation of deamidase activity by TG2 enzyme is strongly increased by the inflammatory tissue process, one question is related to the role of TG activity in the first disease stages of CD both in adults and young subjects with CD. To date, only one study has analyzed the pattern of CD4+T cell reactivity to gluten peptides in a cohort of very young HLA $DQ2^{+}$ Dutch patients (8). This study showed a substantial heterogeneity of intestinal T cell responses to gluten peptides with a large proportion of responses directed to both deamidated and wild type gliadin, and with the identification of six novel epitopes. Importantly, Vader and coworkes showed that for three of these novel epitopes, tTG-mediated deamidation was not required for the cell stimulatory properties, suggesting that, at least in children, the peptide immune activity is independent by deamidation.

Based on these features, it has been hypothesized that in celiac patients, the adaptive T cell response can be primed by naive peptides, and

subsequently, it is driven toward immunodominant deamidated sequences. It could be envisaged that the recognition of few native peptides, at the site of small intestinal mucosa, may trigger the production of inflammatory cytokines, as IFN-γ, and activate an inflammatory loop resulting in tissue damage and TG2 release. As consequence, the $CD4^+$T cell response is strongly enhanced with more IFN-γ production and increased release of TG2. Alternatively, other factors, such as infections occurring in the gastrointestinal tract, may trigger the inflammatory response to naive gluten peptides and the activation of TG2 (8, 10).

In order to investigate the immune response activated in the early stage of disease, a good model may be represented by potential celiac disease. *Potential celiac disease* is a condition characterized by a positive serology for the tissue transglutaminase autoantibodies (tTG2), but a normal morphological duodenal mucosa or with slight signs of inflammation (12). It has been observed that almost 10% of children with positive serology have potential CD and only 33% of them develop the overt disease after 3 years (13). It's not clear if *potential celiac*

disease represents a preclinical condition that affect all celiacs, or if it is different form of disease.

2.1.2 Repertoire of immune-dominant gluten epitopes activating a CD4$^+$ T cell response in adult subjects with celiac disease

Gluten, the trigger factor in celiac disease is a heterogeneous mix of hundreds of proteins that can be divided according to their electrophoresis mobility in gliadin (α–, γ–, ω–gliadins) and glutenin (low and high molecular weight). Gluten proteins are monomers or polymers, these latters linked by inter-chain disulphide bonds, and rich in glutamine and proline, but poor in charged amino acids (14). The wheat genome is high redundant, and consequently in a single wheat variety several hundred different gluten proteins are found. The majority of gluten peptides are involved in the pathogenesis of CD (10). Different research groups, for many years, made a great effort to identify the repertoire of gluten epitopes that play a relevant role in the immune-pathogenesis of celiac disease (9-11, 15-20). These understanding has been possible by the use of long-term T cell lines, and T cell clones

derived from intestinal biopsies of celiac patients; since it has been found that gluten specific $CD4^+$ intestinal T cells can be isolated from intestinal biopsies of celiac patients (9,11,15). These studies have provided the identification of several immunogenic peptides, derived from wheat and from the other prolamines, able to activate specific intestinal $CD4^+$T cells in adult CD patients.

Despite all these results, the great heterogeneity of gluten proteins (14), and technical limitations, in particular the limited number of cells that can be obtained from intestinal biopsies, together with the low frequency of antigen specific cells grown *in vitro*, make very complicated the identification of all gluten immunogenic peptides involved in celiac disease.

To date several immunogenic epitopes from α-, γ-gliadin and glutenin have been identified to activate intestinal $CD4^+$T-limphocytes (8, 9, 16-18) in celiacs.

One of these, a single 33-mer peptide mapping N-terminal 57-89 region of α-gliadin was shown to have the highest T-cell stimulatory capacity in a large cohort of CD patients (LQLQPFPQPQLPYPQPQLPYPQ

PQLPYPQPQPF) (18). This peptide is highly resistant to proteolitic gastrointestinal digestion, and contains six copies of 3 different epitopes DQ2.5-glia-α1a (PFPQPELPY), DQ2.5-glia-α1b (PYPQPELPY), DQ2.5-glia-α2 (PQPELPYPQ) (10). Recently it has been demonstrated that 17-mer peptide (QLQPFPQPQLPYPQPQP), corresponding to truncated form of 33-mer, shows immune-stimulatory similar to the longer peptide. Similarly, it has been identified another 17-mer derived from ω-gliadin DQ2-ω-1, that containing the DQ2.5-glia-ω1 and DQ2.5-glia-ω2 epitopes frequently recognised by $CD4^+$T lymphocyte from $DQ2^+$celiacs from different countries (9, 19).

A large and heterogeneous pattern of immunogenic peptides has been found in the γ-gliadins, although DQ2.5-glia-γ1 shows high reactivity properties in $DQ2^+$ patients (Table 2.1.1) (9, 20).

Epitope	Previous name	Peptide-binding register, P1-P9								
		1	2	3	4	5	6	7	8	9
DQ2.5 restricted epitopes										
DQ2.5-glia-α1a	DQ2-α-I, α9	P	F	P	Q	P	**E**	L	P	Y
DQ2.5-glia-α1b	DQ2-α-III	P	Y	P	Q	P	**E**	L	P	Y
DQ2.5-glia-α2	DQ2-α-II, α2	P	Q	P	**E**	L	P	Y	P	Q
DQ2.5-glia-α3	glia-α20	F	R	P	**E**	Q	P	Y	P	Q
DQ2.5-glia-γ1	DQ2-γ-I	P	Q	Q	S	F	P	**E**	Q	Q
DQ2.5-glia-γ2	DQ2-γ-II	I	Q	P	**E**	Q	P	A	Q	L
DQ2.5-glia-γ3	DQ2-γ-III	Q	Q	P	**E**	Q	P	Y	P	Q
DQ2.5-glia-γ4a	DQ2-γ-IV	S	Q	P	**E**	Q	**E**	F	P	Q
DQ2.5-glia-γ4b	DQ2-γ-VIIc	P	Q	P	**E**	Q	**E**	F	P	Q
DQ2.5-glia-γ4c	DQ2-γ-VIIa	Q	Q	P	**E**	Q	P	F	P	Q
DQ2.5-glia-γ4d	DQ2-γ-VIIb	P	Q	P	**E**	Q	P	F	C	Q
DQ2.5-glia-γ5	DQ2-γ-VI	Q	Q	P	F	P	**E**	Q	P	Q
DQ2.5-glia-ω1	DQ2-ω-I	P	F	P	Q	P	**E**	Q	P	F
DQ2.5-glia-ω2	DQ2-ω-II	P	Q	P	**E**	Q	P	F	P	W
DQ2.5-glut-1	glutenin-17	P	F	S	**E**	Q	**E**	Q	P	V
DQ2.5-glut-2	glutenin-156	F	S	Q	Q	Q	**E**	S	P	F
DQ2.5-hor-1	Hor-α9, Hα9	P	F	P	Q	P	**E**	Q	P	F
DQ2.5-hor-2	Hor-α2, Hα2	P	Q	P	**E**	Q	P	F	P	Q
DQ2.5-hor-3	hor-I	P	I	P	**E**	Q	P	Q	P	Y
DQ2.5-sec-1	Sec-α9, Sα9	P	F	P	Q	P	**E**	Q	P	F
DQ2.5-sec-2	Sec-α2, Sα2	P	Q	P	**E**	Q	P	F	P	Q
DQ2.5-sec-3	DQ2-Sec-I	?								
DQ2.5-ave-1a	Av-α9A	P	Y	P	E	Q	**E**	E	P	F
DQ2.5-ave-1b	Av-α9B, 1490	P	Y	P	E	Q	**E**	Q	P	F

Table 2.3.1: List of celiac disease T cell epitopes recognized by CD4$^+$T cells

[Sollid et al,. Immunogenics 2012 (20)].

Recently, a great boost in this direction was given by a new *in vivo* approach used by Anderson and co-workers, to identify gluten specific T

cells, able to overcame all these problems related to the use of intestinal T cells (21).

This procedure requires the administration to celiac patients on GFD of wheat bread for few days (3 days). Following oral challenge, specific T cells, can be found in peripheral blood of patients, using the sensitive IFN-γ EliSpot assay. This innovative methodology, found reproducible in different cohorts of celiacs (22-24), opens to new tools for investigation of immune pathways activated by gluten, as well as in the clinical practice. By this approach, recently J.A. Tye-Din and co-workers have screened a large library of 20-mer epitopes derived from different gliadin and prolamines as hordein and secalin (19), giving a great contribution to the identification of immunogenic gluten epitopes. A high degree of T cells redundancy was observed among celiacs (21), however three peptides were found responsible of the great majority of T cell responses (DQ2.5-glia-α1a, DQ2.5-glia-α2; DQ2.5-glia-ω-1, DQ2.5-glia-ω-2; DQ2.5-glia-γ-1).

Collectively, these studies have formed the basis for novel a diagnostics and therapies, some of them are currently under clinical investigation.

The comprehension of the T cell immune stimulatory gluten peptides, involved in celiac disease, lead to identification of new therapeutic strategies (25). In the current state, the only treatment for celiac disease is represented by gluten free diet. This dietary consists in the completely avoidance of wheat gluten from the diet. Such dietary treatment allows to ameliorate all lesions of intestinal mucosa, and to normalize serological markers of disease, however it does not guarantee a permanent recovery of disease. In fact, if celiac patients enter in contact with gluten, the intestinal immune cells can be reactivated, resulting in the intestinal mucosa damage.

Furthermore gluten free diet has not a perfect compliance (26% of children and 82,5% of adult show signs of intestinal damage after 2 years of dietary treatment) (4) due to different reasons, particularly for difficulty in finding gluten-free food (26) In addition GFD may creates some issues in social activities related to food. In addition there is a minority of patients that suffers of refractory celiac cases, in which the diet is not efficacious, and require a pharmacological, anti-inflammatory treatment (27).

Celiacs on gluten free diet for many years, can present nutritional deficiency, and could present a significant risk to develop obesity or metabolic syndrome, also those strictly comply this diet (28, 29). These deficiencies could be related to nutritional properties of gluten free products and to the high glycemic index, and caloric density of cereal used, that often are poorer in nutrient levels than naturally wheat grain (28).

For all these reasons identify a new therapeutic strategy to treat celiac disease will be important for a large part of celiac population. One strategy currently under investigation is peptide-based immunotherapy, already used to treat other auto-immune disease or allergies (30, 31).

Peptide based immunotherapy aims to immune-reactivity modulation, by induction of tolerogenic response, and elimination of the inflammatory state (32). It has been demonstrated that subcutaneous or sublingual administration of immunogenic peptides, activates the tolerogenic immune pathways. The results of peptides based-immunotherapy are modulation of immune-reactivity, with down-regulation of the immune

response and stimulation of regulatory T cells. A clear application of this method is represented by the vaccines (30, 33).

In order to design a potential immunotherapy for CD, the detailed comprehension of the T-cell stimulatory epitopes, and of their possible immune-dominance, is necessary.

A current perspective of peptide-based therapeutic vaccine in celiac disease is represented by Nexvax2. This drug includes three epitopes that results immunodominant in HLA-DQ2 celiacs. Currently Nexvax2 is on Phase II clinical trial in adult celiac patients in both Australia and USA, in order to assess its efficacy in controlling of inflammatory state (34). However, the feasibility of such applications will depend upon consistency of the immune response to gluten peptides despite the gluten cereal consumed, duration of disease and age.

2.2 AIM

Since the immune response to gluten in children with CD is poorly studied, this project aims to provide an understanding of the T cell immune-stimulatory gluten peptides involved in childhood celiac disease. Furthermore, we have also investigate the role of the deamidation by tTG on the stimulatory properties of gluten peptides in children with *classical* and *potential* celiac disease by looking at intestinal $CD4^+$T cell response to both native and deamidated gluten.

This project comprises the following experimental phases:

- Analysis of immune reactivity to known immunodominant gliadin epitopes in children with overt celiac disease.
- Analysis of adaptive, $CD4^+$Tcell-mediated responses in the intestinal mucosa of children with potential celiac disease
- Identification of immunogenic gliadin peptides in potential celiac disease
- Investigation of the role of the deamidation by tTG on the immune recognition of gluten in the intestine of very young subjects with CD.

2.3 MATERIALS AND METHODS

2.3.1 Selection and synthesis of gluten peptide library and control antigens

Peptic-tryptic digestion of gliadin (PT-gliadin) has been obtained following extraction from Strampelli grains as elsewhere destribed (14), and enzymatically digestion with pepsin and trypsin (35). Gliadin peptides (Table 2.3.1) were synthesized by solid-phase automated flow, as previously described (9). Both gliadin and peptides were deamidated with guinea pig tTG (Sigma- Aldrich, St. Louis, MO, USA), as reported elsewhere (9).

This phase of project was performed in collaboration with researchers of Institute of Food science-CNR, Avellino.

Cod	Peptide Name	Sequence	Reference
P1	DQ2.5-glia-α1a, -α2	QLQPFPQPQLPYPQPQP	Camarca, JI 2009 (9)
P2	DQ2.5-glia-ω-1,-ω-2	QPQQPFPQPQQPFPWQP	Camarca, JI 2009 (9)
P3	DQ2.5-glia-γ-1	PQQPQQSFPQQQQPA	Camarca, JI 2009 (9)
P4	DQ2.5-glia-γ-2	GIIQPQQPAQL	Camarca, JI 2009 (9)
P5	DQ2.5-glia-γ3;-γ4b and -γ4c;-γ5; glia-γ-2	FLQPQQPFPQQPQQPYPQQPQQPFPQ	Shan et al Protome Res 2005 (36)

Table2.3.1: Gliadin peptides analyzed in this study

2.3.2 Recruitment of population study

Children were enrolled at "Section of Pediatrics", at the Department of Translational Medical Science, University of Naples "Federico II". Children were enrolled based on suspected CD or potential celiac disease on the basis of positive CD serology (anti-endomysial antibodies (EMA) and/or of anti-tissue transglutaminase (anti-tTG) antibodies). All children who received CD diagnosis were retained in the study, and with the parents' informed signed consent to the experiments.

	Age/sex	TTG	Histology	DQ	DR
HC20	10/F	<7	T0	DQ2/DQ8	DR3/DR4
HC21	12/M	<7	T1	DQ8/DQX	DR4/DR13
HC22	15/M	<7	T0	DQ2/DQX	DR3/DR9
HC23	6/M	<7	T1	DQ8/DQX	DR4/DR16
HC24	15/M	<7	T0	DQ2/DQ2	DR3/DR3
HC25	14/M	<7	T0	DQ2/DQX	nd
Pot1	10/F	<7	T1	DQ2/DQ8	DR3/DR4
Pot2	24/F	<7	T0	DQ8/DQX	DR4/DR16
Pot3	12/F	30	T1	DQ2/DQ8	DR3/DR4
Pot4	5/M	12.4	T1	DQ2/DQX	DR1/DR3
Pot5	10/F	14.5	T1	DQX/DQ8	DR1/DR4
Pot6	10/F	8.7	T0	DQ2/DQ8	DR3/DR4
Pot7	4/F	23.7	T1	DQ2/DQ7	DR5/DR7
Pot8	15/M	9.3	T1	DQ2/DQX	nd
Pot9	10/M	<7	T1	DQ2/DQX	nd
Pot10	4/M	21.5	T1	DQ2/DQ2	DR3/DR3
Pot11	9/F	11.9	T1	DQ2/DQ2	DR3/DR3
Pot12	11/M	<7	T0	DQ2/DQX	nd
Pot13	17/F	<7	T1	DQ2/DQX	nd
Pot14	11/F	<7	T0	DQ2/DQX	DR3/DRX
Pot15	10/F	<7	T1	DQ2/DQX	nd
Pot16	13/M	<7	T0	DQ2/DQX	nd
Pot17	15/F	23	T1	DQ2/DQX	nd
CD1	18/M	22	T3b	DQ8/DQX	DR4/DR13
CD2	1/F	78	T3c	DQ8/DQX	DR4/DR13
CD3	6/F	300	T3b-c	DQ2/DQ2	DR3/DR3
CD4	14/F	200	T3c	DQ2/DQX	DR1/DR3
CD5	14/M	100	T3c	DQ2/DQX	nd
CD6	13/M	138.6	T3c	DQ2/DQX	nd
CD7	11/m	203	T3c	DQ2/DQX	nd
CD8	9/M	47	T3b/c	DQ2/DQ7	DR3/DR5
CD9	15/F	<7	T3a	DQ2/DQ7	DR3/DR5
CD10	15/M	15.9	T3b	DQ2/DQX	nd
CD11	9/F	291	T3b/3a	DQ2/DQX	nd
CD12	15/M	27	T3a	DQ2/DQX	nd
CD13	9/F	159	T3b	DQ2/DQX	nd

Table 2.3.2: List of Children enrolled in this study

2.3.3 Isolation of PBMC from peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised whole blood using Ficoll (Ficoll-PaqueTM PLUS-GE Healthcare, UK) gradient separation.

2.3.4 Generation of gliadin-specific, polyclonal T cell lines

All children enrolled underwent to Jejunal biopsies. This biopsies of about 3-4 mm in size, were enzymatically digested with collagenase-A (1mg/ml, Roche, Basel, CH), for 1hour at 37°C and 5% CO_2. Isolated cells were stimulated with irradiated autologous PBMCs, and deamidated gliadin (25µg/ml) or both deamidated and naive gliadins (25µg/ml) used as antigen.

48-hours later stimulation, were added 1ml of fresh medium, and IL-15 (10ng/ml) and IL2 (40U/ml) (R&D System, Minneapolis, MN). At day 7, T-cell lines (TCLs) generated were re-stimulated once more with autologous PBMCs and PT-gliadin±tTG; IL-15 and IL2 were added after 48-hours and every subsequent 3-4 days. Long term TCLs were generated by cycling of restimulation with Phytohaemagglutinin (Roche, Basel, CH) and heterologous PBMCs.

2.3.5 T cell Assay

T cell lines generated from intestinal biopsies were assayed in response to gliadin and gliadin peptides. T cells were seeded in a 96-well plate, autologous or HLA matched EBV were used as APC (antigen presenting cells), gliadin (50μg/ml) or gliadin peptides (30μg/ml) were used as antigens. Cells were incubated at 37°C and 5% CO_2. 48-hours later, supernatant, derived from T cell assay, were collected and used to assayed IFN-γ levels using Elisa Assay.

2.3.6 IFNγ ELISA assay

A 96-well high binding (Corning, NY) plates were coated with anti-human (1μg/ml) IFN-γ mAb (Mabtech, OH) overnight at 4°C. Thereafter, plates were washed (PBS-Tween 20-Sigma-Aldrich, St. Louis, MO), and blocked with blocking solution (PBS+2%BSA-Sigma-Aldrich, St. Louis, MO) for 2-hours at room temperature. Cell supernatants, previously collected by T-cell assay, were added to plate overnight at 4°C.

Thereafter, plate were washed, and incubated with biotinylated anti-IFN-γ (Mabtech, OH) at 1 μg/ml, 1-hour at room temperature, and subsequent incubated with Streptoavidina HRP (BD-Pharmingen, Franklin Lakes, NJ) in a diluition buffer. The addition of 3,3',5,5'-tetrametilbenzidin (TMB, Sigma-Aldrich, St. Louis, MO) diluted in a Substrate buffer (citric acid monohydrate pH4.2) allow to quantified IFN levels produced using spectrophotometer.

2.3.7 IFN-γ EliSpot assay

Peptides specific T cells responses were observed usingγ-IFN EliSpot Assay. T cells ($3x10^4$) were seeded on a 96 wells nitrocellulose backed plate (MAHA S4510- Millipore, Bedford, MA), previously coated with anti (10μg/ml) IFN-γmAb (Mabtech, OH) over-night at 4°C. Autologous or HLA matched EBV were used as APC (antigen presenting cells), and gliadin (50μg/ml) or gliadin peptides (30μg/ml) were used as antigens. Cells were incubated 48-hours at 37°C and 5% CO_2.

Following cells incubation, plates were treated with biotinylated anti-IFN-γ (Mabtech, OH) 2-hours at room temperature. Thereafter plates were washed with PBS-0,05% Tween20 (Sigma-Aldrich, St. Louis, MO) and then incubated with Streptoavidina HRP (BD-Pharmingen, NJ) in a dilution buffer (PBS1X/BSA1%, Sigma- Aldrich) for 1h at room temperature. The addition of aminoethylcarbazole (AEC, Sigma-Aldrich) solution for five minutes, make detected cytokines releasing cells by using ELISPOT image analysed (A.EL.VIS.)

2.4 RESULTS

2.4.1 Recognition of known immunodominant gliadin peptides in children with classical form of celiac disease

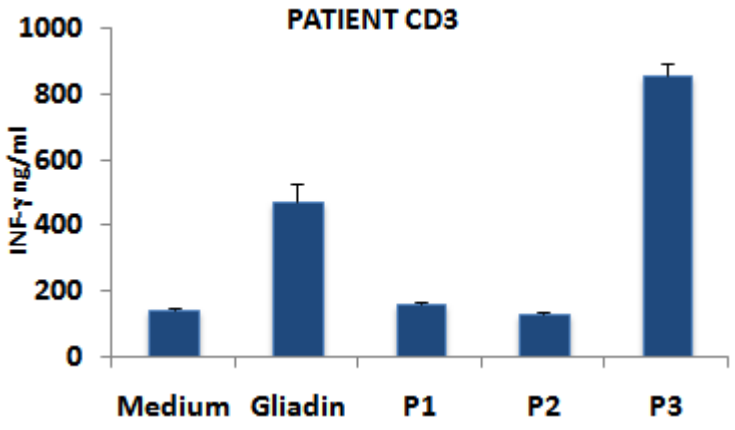
As above mentioned, the identification of gluten epitopes plays an important role in the characterization of celiac disease. Up to now a large panel of gliadin epitopes has been found immunogenic for adult CD patients. In order to characterize the involvement of the main of these peptides (listed in Table 2.4.1) in children form of disease, jejunal biopsies were collected, and T cell lines generated from children with overt celiac disease.

Cod	Peptide Name	Sequence
P1	DQ2.5-glia-α1a, DQ2.5-glia-α2	QLQPFPQPQLPYPQPQP
P2	DQ2.5-glia-ω-1, DQ2.5-glia-ω-2	QPQQPFPQPQQPFPWQP
P3	DQ2.5-glia-γ1	PQQPQQSFPQQQQPA

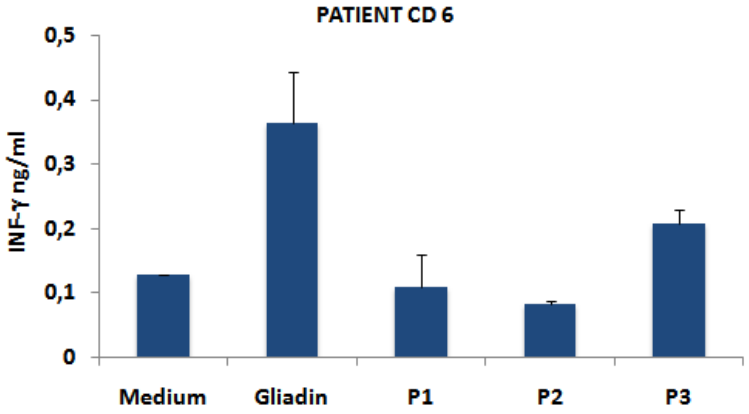
Table2.4.1: Panel of gliadin peptides assayed in children celiac

Specific reactivity to deamidated-peptides generated by T cell lines was measured using IFN-γ ELISA/EliSpot assays. Peptic-tryptic digest of gliadin has been used as control.

a)



b)



c)

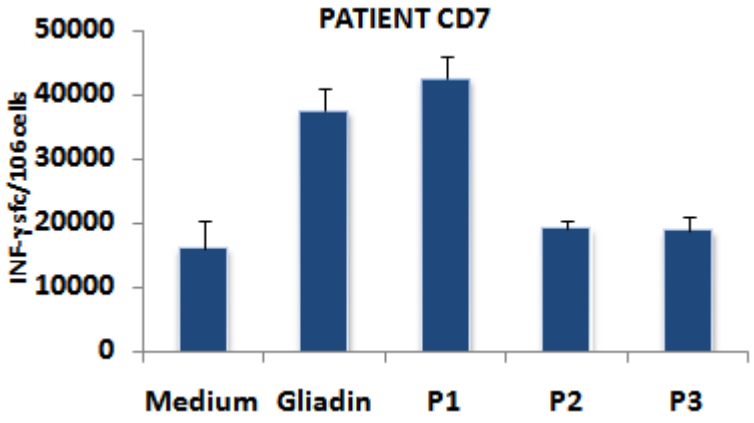


Figure 2.4.1: Specific responses to Pt-gliadin and gliadin peptides in celiac children CD3 a), CD6 b) CD7 c)

In figure 2.4.1 we have shown representative results obtained in three celiac children. Patient CD3 (part a) has shown very high production of IFN-γ in response to P3 (DQ2.5-glia-γ1) peptide, and low production in response to P1 and P2 (DQ2.5-glia-α1a/glia-α2; DQ2.5-glia-ω-1/glia-ω-2). Similar pattern of reactivity has been foun in patient CD6.

In contrast, patient 7 have reported very high levels of IFN-γ secreting cells in response to P1 (DQ2.5-glia-α1a/glia-α2) peptide, and only weak levels in presence of P2 and P3 peptides (DQ2.5-glia-ω-1/-glia-ω-2; DQ2.5-glia-γ1).

All T cell lines have shown reactivity to PT-gliadin.

Collectively our data have indicated that T cell lines generated from children celiac recognized same gliadin peptides specifically identified in adult celiac patients. Furthermore, a great heterogeneity in reactivity to these peptides was found; with no prevalence response generated by one or more peptide(s). Similar pattern of responsiveness was previously found in adult affected by celiac disease (9).

2.4.2 T-cell mediated immune response to gliadin in potential

In order to further improve comprehension of early phases of celiac disease, we decided to identify immune-responses to PT-gliadin in children celiac patients. We have decided to investigate gliadin reactivity found in T cell lines established following children intestinal biopsies; and in particular we have focused on immune responses obtained in potential celiac patients.

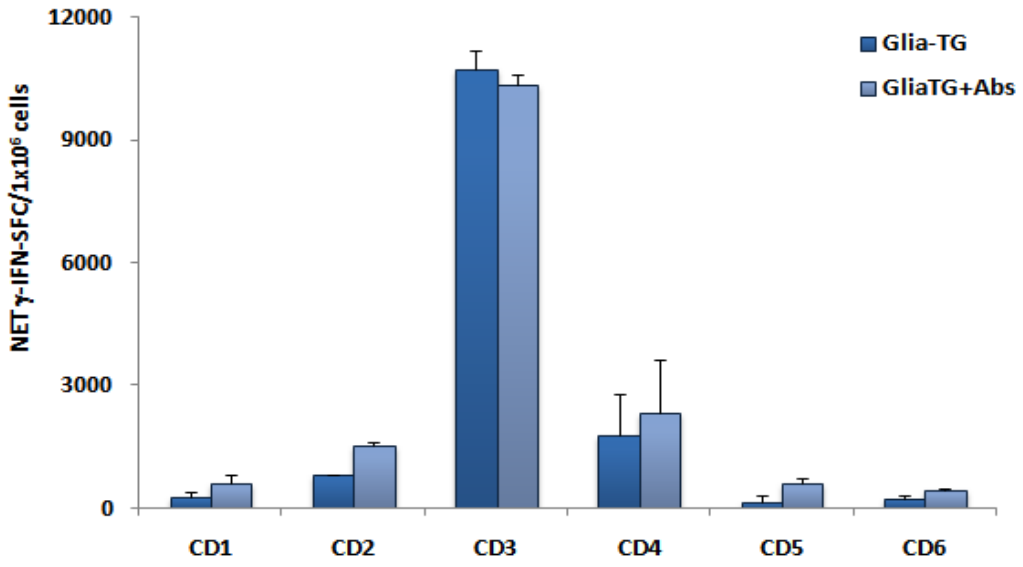
We enrolled a population study composed by potential, celiac children, and non celiac controls. Using EliSpot Assay we measured IFN-γ secretion cells activated by deamidated PT-gliadin. Results were expressed as Net-γ-IFN-SFC/10^6cells. Furthermore T cell lines were assayed with and without presence of antibodies neutralizing, in order to investigate the involvement of regulatory pathways in controlling the GALT responses to dietary gluten (37). We consider positive response that presented IFN-γ levels in presence of gliadin at least 2 value of medium alone.

In figure 2.4.2 we showed results obtained. In part A) we have represented responses from six children with acute disease (positive TTG and with villous atrophy). Results obtained were heterogeneous. A part of children celiac subjects have shown a positive production of IFN-γ in response to gliadin in at least one experimental condition (with or without neutralizing antibodies).We have found patients showing a significant response both with and without neutralizing Ab, or only with neutralizing Ab. Particularly patient 6 presents a positive response both with and without neutralizing antibodies. Similarly patient 1 and 5 present significant responses only when were present neutralizing antibodies, and present borderline values in absence of these. Other two patients have shown border line values (1.8-1.9 values of medium alone) when we added neutralizing antibodies.

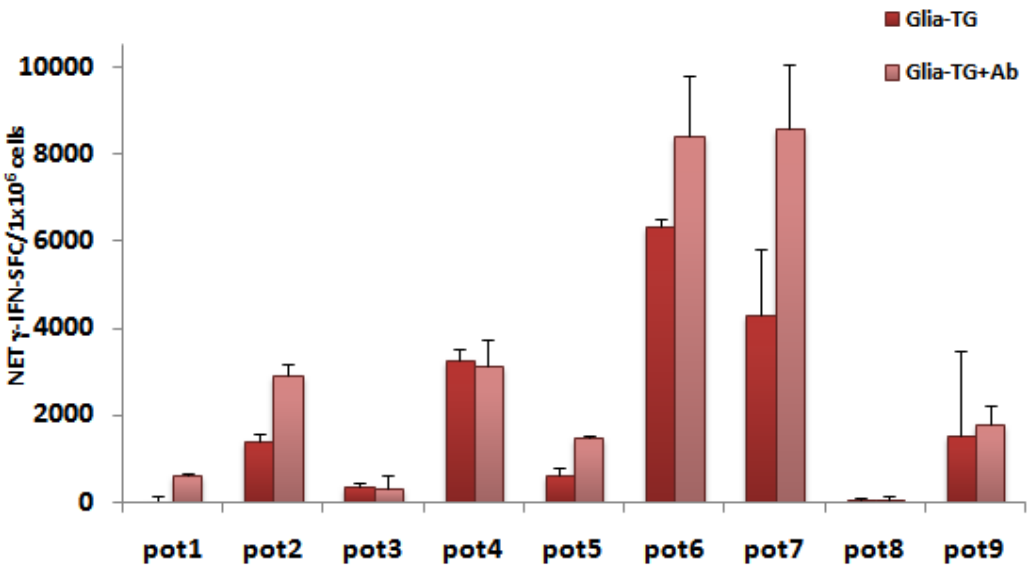
Overall these results highlighted a heterogeneous pattern of reactivity to gliadin, but generally there is an increase in IFN-γ production in presence of neutralizing antibodies.

In part B) we have shown results obtained thought IFN-γ EliSpot Assay in 9 children with positive TTG and with normal mucosa. We considered

these subjects as potential celiac. We assessed these T cell lines both in presence or absence of neutralizing antibodies. In our results we have found that 2 potentials (pot 2, 4,) presented a significant response to gliadin both with and without neutralizing antibodies, while patients 7 and 9 only in presence of neutralizing antibodies. In addition we have found two subjects presenting borderline values (1.8-1.9 values of medium alone) when were added neutralizing antibodies, while one of subjects both whit and without neutralizing antibodies.



A)



B)

Figure.2.4.2 Gliadin specific T cell responses in children celiac (A), and potential celiac (B) with or without neutralizing antibodies

Overall these data have demonstrated that along with T cell responses found in celiac children, we identified a clear reactivity to deamidated gliadin generated by T cell lines of potential celiac. Furthermore we have shown that both in acute and potential children celiac, the adaptive response to gliadin in children CD could be under control of regulatory pathways.

2.4.3 Comparative analysis of gliadin-specific T-cell responses

In order to investigate the immune-responses generated, and gliadin specific reactivity found in potential celiac, we compared the responses found in these children to responses obtained in non celiac controls, and in children celiac patients. In figure 2.4.3 are shown results obtained by using ElisSpot IFN-γ assays in response to deamidated form of gliadin. Results were expressed as Net IFN-γ SFC/10^6 cells (SFC in the presence of Gliadin-TG minus SFC in absences of Gliadin).

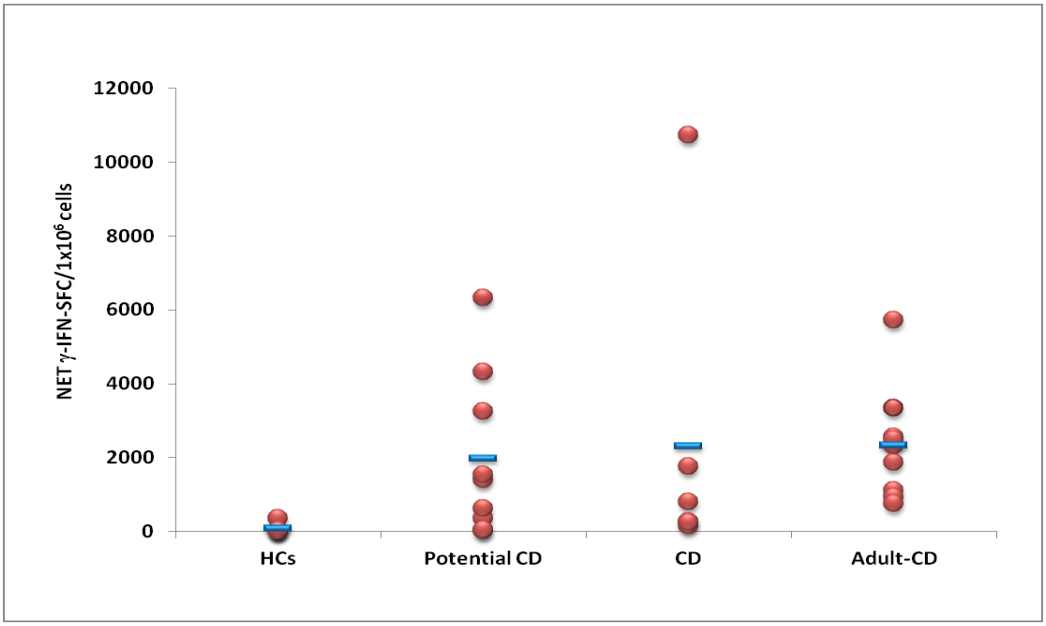


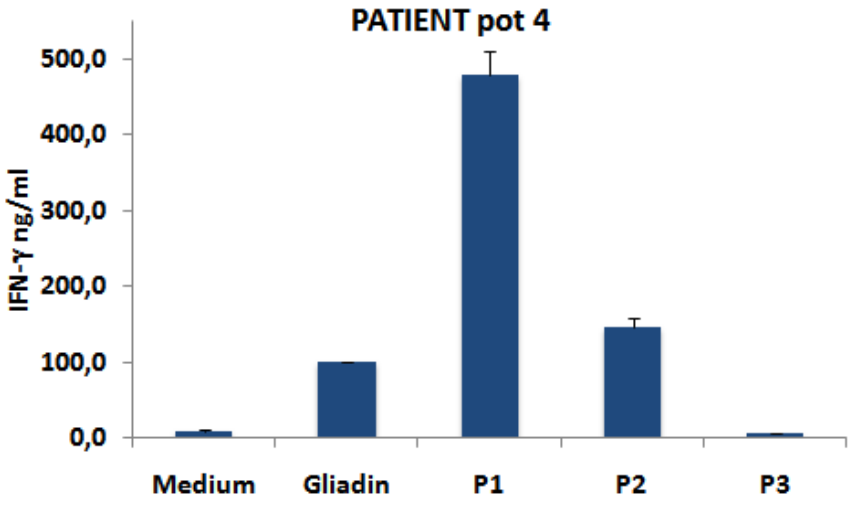
Figure2.4.3: Comparative analysis of T cell lines derived from HCs, Potential, Children and Adult celiacs

Our results have shown that no significant differences in IFN-γ production in response to PT-gliadin has been found, among potential-CD, and both adult (p=0,69) and children celiac patients (p=0,86). In contrast, a great difference [(T-Test student (p<0,03)] have been found, when responses found in potential-CD were compared with responses of non celiac subjects.

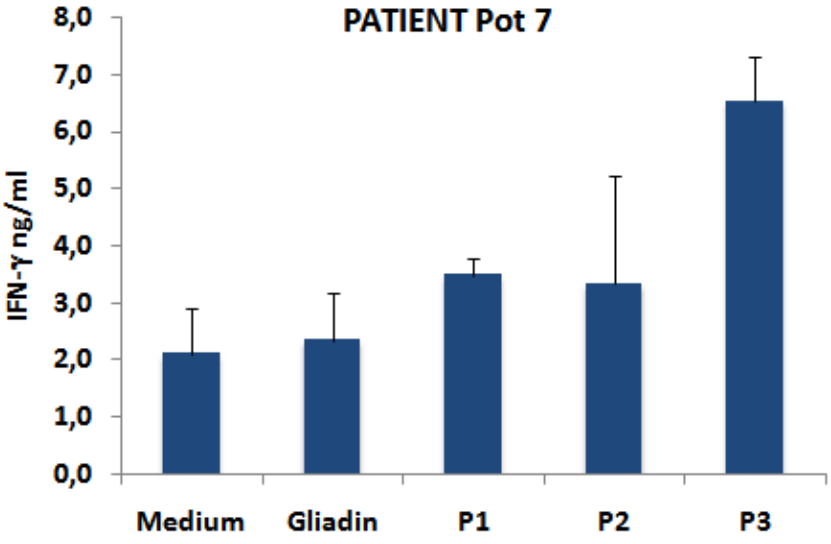
2.4.4 Recognition of adult immunodominant gliadin peptides by children with potential celiac disease

We have already assessed the presence of gliadin specific T cells in potential celiac. In order to expand the investigation on potential celiac disease, we decided to assayed reactivity generated by main immunodominant gliadin epitopes, previously assessed in children celiac (Table 2.4.1) subjects.

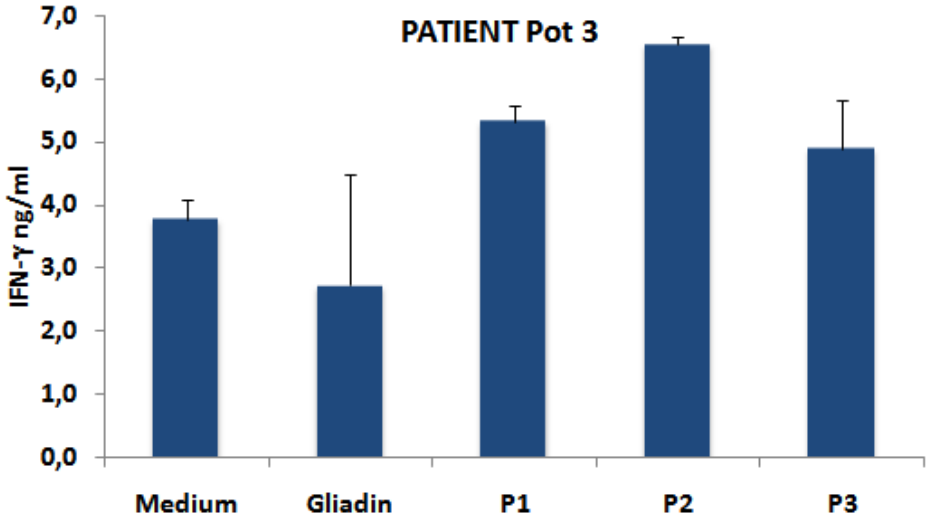
Results were obtained using Elisa Assays, and were shown as IFN-γ ng/ml. We considered responsive all these T cell lines that show IFN-γ levels in response to peptides at least 2 value of medium alone.


PATIENT pot 4
IFN-γ ng/ml
500,0
400,0
300,0
200,0
100,0
0,0
Medium
Gliadin
P1
P2
P3

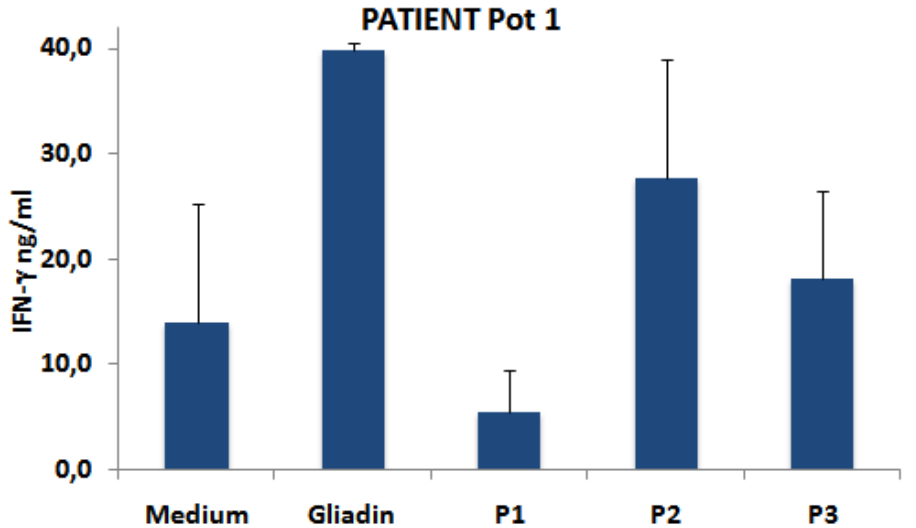
A)


PATIENT Pot 7
IFN-γ ng/ml
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Medium
Gliadin
P1
P2
P3

B)



C)



D)

Figure 2.4.4: Specific T cell responses from potential celiac patients to selected gliadin peptides

In figure 2.4.4 is shown the representative immnune-responses to gliain peptides generated by T cell lines of potential children. Patient potential

4 presented very high INF-γ levels production in presence of P1 and P2 (DQ2.5-glia-α1a/glia-α2; DQ2.5-glia-ω-1/glia-ω-2), no positive response has been found in response to P3 (DQ2.5-glia-γ1).

In contrast, patient potential 7 has demonstrated a increased IFN-γ secretion in presence of all assessed epitopes, however only in presence of P3 (DQ2.5-glia-γ1), the response was clearly high. Similary, to potential 7, also potential 3 presented increase in IFN-γ production in presence of all selected peptides. However we have identified a very high response only when is present P2 peptide (DQ2.5-glia-ω-1/glia-ω-2).

Finally in patient Potential 1we found a very high IFN-γ production in response to P2 (DQ2.5-glia-ω-1/glia-ω-2).

Overall these results have shown that potential celiac have T cells able to recognized main immune-dominat gliadin peptides with a high heterogenteity. All peptides have shown immunoreactive properties, and we have not found a dominant response by one or more peptides(s).

2.4.5 T-cell mediated immune response to gliadin in potential children

Recently, one hypothesis occurs, suggesting that in first stages of disease, the T cells activation could be start in response to naive gliadin peptides. The consequent activation of inflammatory state could induce the activation of TG2 enzyme and follow deamidation of gliadin peptides. This state results in a further induction of T cells that could be driven toward immunodominant deamidated sequences of gliadin.

To investigate the primary immune responses involved in first stages of celiac disease, and role of TG deamidation, we assayed gliadin reactivity of T cell lines derived from celiac and potential children. Particularly we measured IFN-γ by EliSpot/Elisa assays in response to gliadin, both in naive and deamidated form. The experiments were performed in presence of antibodies neutralizing the effect of IL-10 and TGF-β (10μg/ml) added 10 minutes before antigens, in order to investigate the involvement of regulatory pathways (37).

A total of fourteen TCLs were generated and assayed. We consider responsive all these T cell lines that showed IFN-γ levels in response to gliadin at least 2 the value of medium alone.

In figure 2.4.5 were shown results obtained in potential celiac. These results were expressed as fold increase (IFN-γ produced in absence of antigen/IFN-γ produced in presence of antigen).

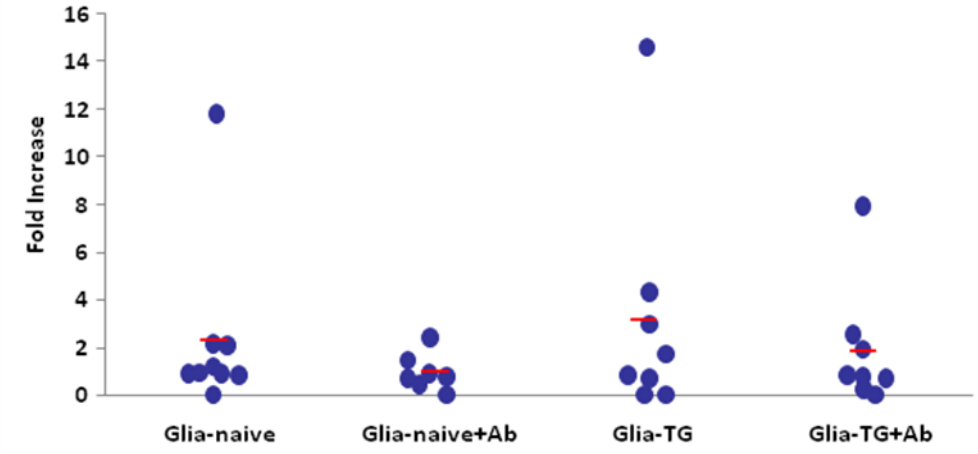


Figure 2.4.5: Responses to Gliadin Naive and Deamidated in potential celiac. Red Bars shown mean values found.

Results (fig. 2.4.5) have shown a heterogeneous pattern of response, in particular in three children the responses to naive PT-gliadin exceeded cut-off value, but only in one of these the positivity was confirmed in presence of neutralizing antibodies. Furthermore three patients expressed

a positive T cell response in presence of deamidated gliadin, and two in presence of neutralizing antibodies in three of them.

Also we have investigated the immune reactivity to gliadin by TCLs generated from six young classical celiac. We considered as classical celiacs children that after endoscopy show intestinal mucosa damage (T3).

As shown in figure 2.4.6 in four children the immuno responses resulted positive at least in one experimental condition. Three children responded to gliadin with and without neutralizing antibodies, three were responsive in presence of deamidated gliadin, while four in response to deamidated gliadin with neutralizing antibodies.

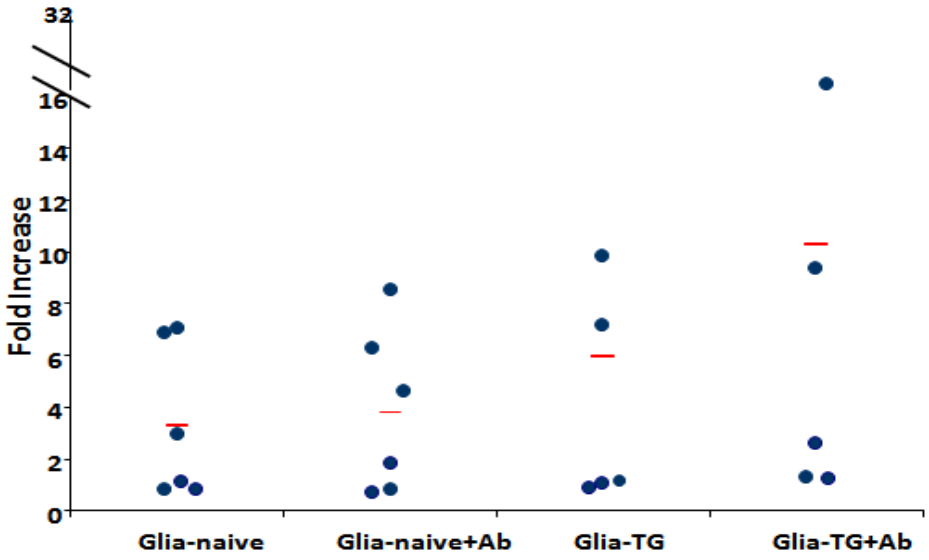


Figure2.4.6: Specific T cell responses to gliadin Naïve and Deamidated in celiac children. Red bars shown mean value.

The trend of the responses that we have identified shown a growing increase in the IFN-γ production in response to both naive and deamidated PT-gliadin. This positivity has been found accentuated, in progressive manner, in presences of neutralizing antibodies. The amount of increase was generally higher in response to pt-naive than deamidated gliadin.

Overall these data indicated that both potential and celiac children have present T cells reactive both to naive and deamidated gliadin. Generally the presence of neutralizing antibody caused an increase in IFN-γ production, highlighting that regulatory pathway could play a role in the regulation of adaptive T cell response.

2.4.6 T-cell mediated immune response to gliadin peptides

Previously we have investigated reactivity of T cells derived from celiac children to TG-epitopes. To extend our knowledge about role of deamidation in the onset of disease, and on role of gliadin peptides we evaluated reactivity to five epitopes identified previously immunodominant in adult affected by celiac disease.

Cod	Peptide Name	Sequence
P1	DQ2.5-glia-α1a, -α2	QLQPFPQPQLPYPQPQP
P2	DQ2.5-glia-ω-1,-ω-2	QPQQPFPQPQQPFPWQP
P3	DQ2.5-glia-γ-1	PQQPQQSFPQQQQPA
P4	DQ2.5-glia-γ-2	GIIQPQQPAQL
P5	DQ2.5-glia-γ3;-γ4b and -γ4c;-γ5; glia-γ-2	FLQPQQPFPQQPQQPYPQQPQQPFPQ

Table 2.4.2: Complete list of gliadin peptides assayed

In addition we assessed these peptides both in naive and deamidated form. In all experiments Pt-gliadin has been used as control.

In the following figures were have shown the responses to selected peptides. Results have been presented as IFN-γ ng/ml. We considered positive responses that exceeding 2 value of medium alone.

CD#11 presented a very high response to P4 both in naive and deamidated form. Lower positive response was found towards P2, P3, P5, and to naive form of P1.

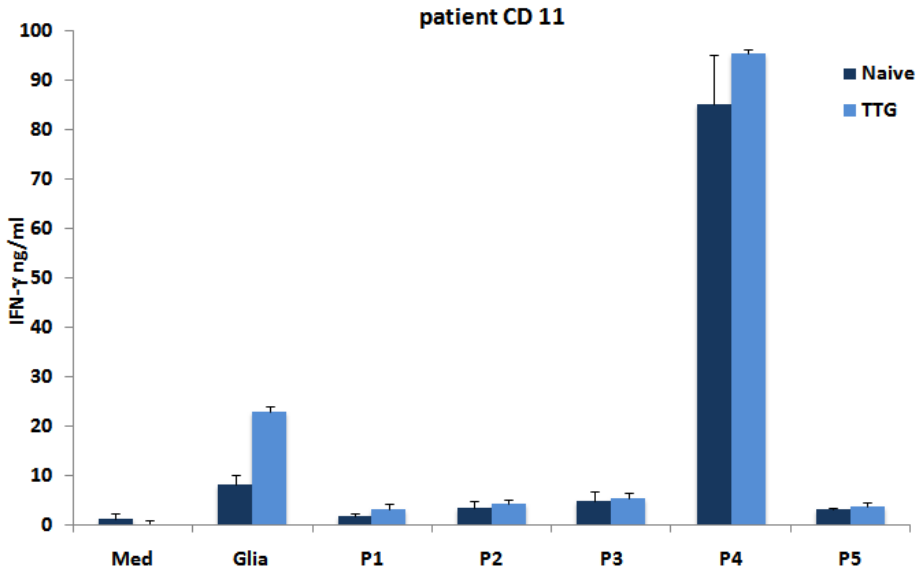


Figure 2.4.7: Responses obtained in patient CD11 in response to gliadin peptides in naïve and deamidated form

CD#12 has shown a positive response to P1 (DQ2.5-glia-α1a/glia-α2) and to P4 in naive and deamidated, while no significant responses have been found in response to other peptides.

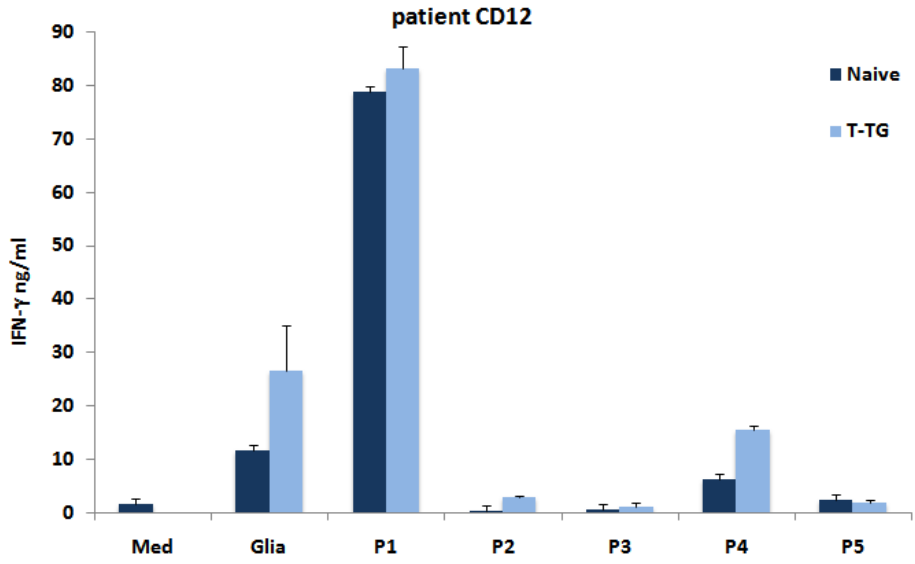


Figura 2.4.8: Responses obtained in patient CD12 in response to gliadin peptides in naïve and deamidated form

Patients CD#9, has shown immune reactivity especially in response to deamidated form of gliadin peptides. In particular significant levels of IFN-γ were identified in response to deamidated P2 and P5 peptides.

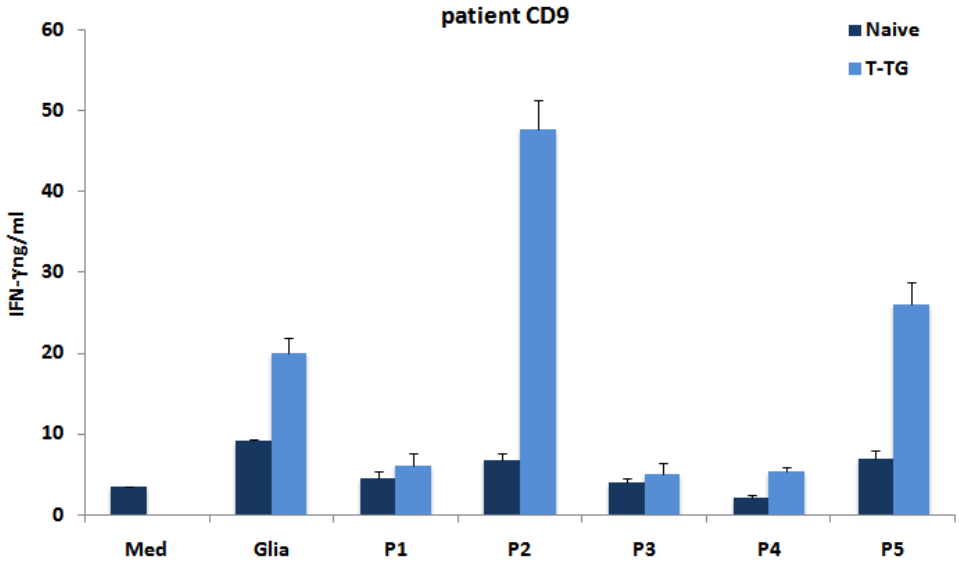


Figure 2.4.9: Responses obtained in patient CD9 in response to gliadin peptides in naïve and deamidated form

Furthermore we have shown results obtained in patient CD#13, we identified a clear increase of IFN-γ production in response to P1(DQ2.5-glia-α1a/glia-α2) and P2 in naive form, we do not confirm these responses in deamidated form. In contrast we identified an immune response to deamidated P4 and P5 peptides.

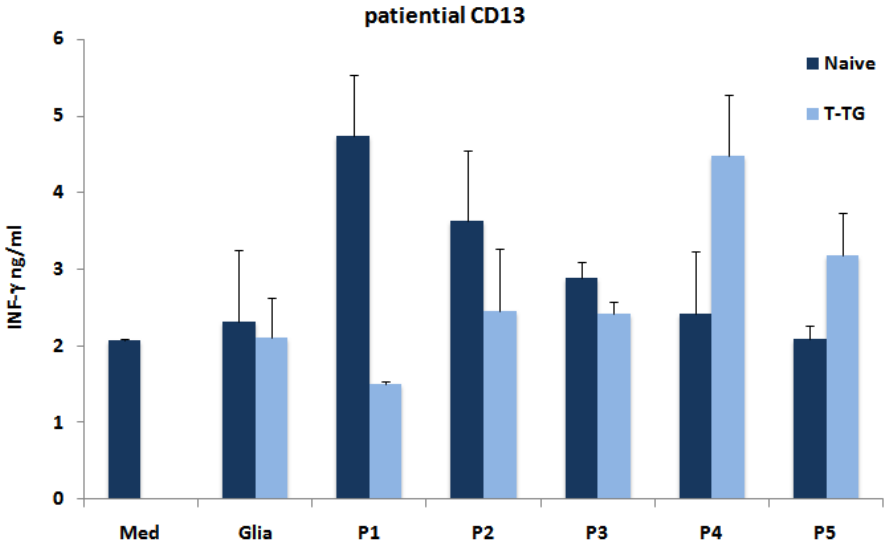


Figure 2.4.10: Responses obtained in patient CD13 in response to gliadin peptides in naïve and deamidated form

In the following figure we have displayed the profile of responses to different peptides, in our patients. Overall we found that all patients produced IFN-γ in response to almost one naive and deamidated peptides.

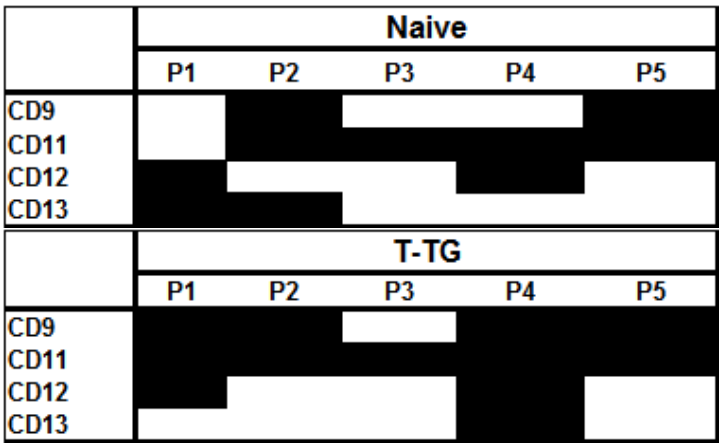


Table 2.4.4: Profile of peptide recognition in children patients

A total of two patients have been found responsive to naive form of P1, rising up to three with deamidated form. Furthermore we have found that three patients produce specifically IFN-γ in response to naive form, two in presence of deamidated form. Only one child has responded both, to naive and deamidated form of P3. Also, we have identified two patients' responders to naive P4, four to deamidated form. Finally we can see that two patient responded to P5 naive confirming responses to P5-deamidated.

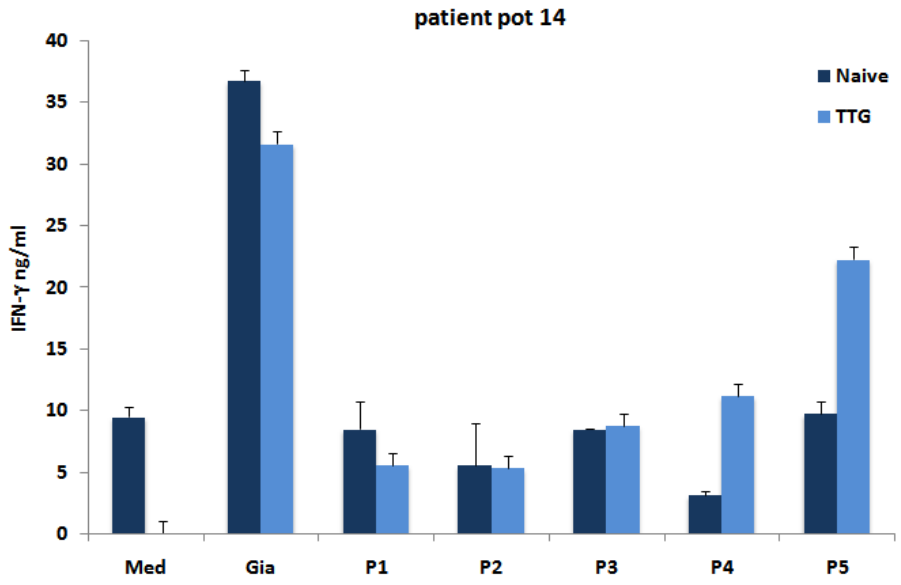


Figure 2.4.11: Responses obtained in potential 14 in response to gliadin peptides in naïve and deamidated form

Finally to have first indication on the involvement of these peptides in potential deamidation we showed results obtained in potential #14. In this child in addition to a significant response to pt-gliadin, we found a clear reactivity to P5 in deamidated form.

Overall these data have demonstrated that children CD patients, and by first indication also potential patients recognized immunodominant peptides. The responses were heterogeneous, since no immune-prevalence of one or more peptides has been found. Furthermore naive

form of peptides have been similarly recognized, and with a similar magnitude in IFN-γ secretion.

2.5 DISCUSSION

Calic Disease is a gluten (or releated prolamines) mediated enteropathy, that affects genetically susceptible individuals carrying HLA class II DQ2 or DQ8 (38).

It has been widley demonstrated that CD presents a wide spectrum of clinical manifestation, that often makes difficult the identification of all affected patients. Among different forms of celiac disease it's possible to identify children with histologially normal mucosa, and positive antibodies. Ferguson have defined these patients potential celiacs (39).

Over the years, the number of publications about celiac disease has increased enormously to elucidate all clinical and immunological characteristics of CD. Despite that, currently, the only effective treatment for CD is gluten free diet. The use of these treatment is widley accepted for classic celiacs, however and its use is under debate in potential celiac (13, 40-43).

Further studies aimed to elucidate the repertoire of pathogenic gluten epitopes immune-dominant in celiac disease, and the network of cytokines produced following the activation of T-cells. These

information provide important tools for diagnosis of disease, and for engineering of alternative therapy such as peptide-based immunotherapy (31, 32)

Recently Camarca and co-workers have assessed the specific immune-reactivity of twenty-one peptides, derived from gliadins and glutenis, by using polyclonal T cell lines from $DQ2^{+}$ celiac adult subjects. Results show a high heterogeneity of responses to these epitopes, and revealed the possible involvement of γ- and ω- gliadins for CD pathogenesis (9).

In our project we extended these analysis to children with classical celiac and potential celiac disease. Since a complete study of large gluten peptides library in pediatric CD is feasible, for the reduced amount of blood draw, we assessed in first instance three peptides derived from α-, ω-, and γ-gliadin, found immune-dominant in adults with celiac disease, and investigated the contribution to the gluten-specific T cell response, in childhood CD. Surprisingly, our results show that intestinal T cell lines from celiac children were specifically activated by same set of gliadin immune-dominant epitopes recognized by adults celiac. No prevalence of one peptide-specific response was found, and furthermore

our patients gave a heterogeneous array of responses. Similar results have been found also in adults (9).

As expected our data confirmed presence of reactive T cells in villous atrophy in celiac children (8). Remarkable, these results show a clear presence of gliadinTG specific T cells in a part of potential-CD. Unexpectedly we found a significant IFN-γ production also in patients whose tTG antibody-titer were fluctuating, and it seems that does not depend on Marsh grade (two T1 and one T0). The low frequence of reactive T cells derived from children found in this study, may be because difficult to *in vitro* expanding and maintaining gliadin specific T cells following polyclonal stimulation with PHA. Previously studies reported that a part (35,3%) of potential celiac develops villous atrophy over time (3 years) (13), also sign of T-cell activation, with increased $\gamma\delta^+$ cells have been identified (12, 43).

Recently it has been highlighted that a large proportion of potential celiac patients, despite persistent positive anti-TG2, don't develop intestinal damage after 9 years of follow-up. The Authors have identified 3 Single Nucleotide Polymorphisms (SNP) of genes (previously

associated to celiac disease) that contribute to predict cases who develop intestinal lesion. All these genes were involved in the pathogenesis of the gluten-induced damage (Auricchio R. et al. *in press* American Journal of Gastroenterology.2014). The expression of one of these genes IL2-IL21 gene has been previously studied in potential celiac disease. In particular it has been found suppressed in potential phenotype (44). Overall these data highlighted that regulation of these gene is associated to pathogenesis of CD.

Furthermore by *ex-vivo* study Westerholm-Ormio and coworkers, reported that density of IFN-γ cells in epithelium of potential celiac patients was found higher than the density of controls, and was found higher in overt CD than potential patients. Our findings had shown no statistically difference in magnitude of IFN-γ production among potential and celiac (45) patients. Overall our data results are important to our knowledge, because they described the presence of this cytokine in potential celiac patients. This is important to investigate the possible start up of inflammatory process which is required to develop overt CD.

Furthermore, in our study all pattern of responses investigated, both in potential and overt celiacs were regulated and influenced by regulatory pathway. All responses were modulated in presence of antibodies neutralizing IL10R and TGFβ, two citokynes involved in controlling of Treg cells. Several studies have suggested that the immunosuppressive cytokines such as IL-10 and TGF-have an important role in maintaining intestinal tolerance both in mouse and human (37, 46-49). Our data were consisitent with previously published data that suggest presence of Treg1 in intestinal mucosa of celiac patients (37), and confirmed role of regulatory system in celiac and in potential celiac disease. Tr1 cells down-regulate naive and memory T cell responses upon local secretion of IL-10 and TGF-(β). In concordance with this hypothesis we identified an increase in IFN-γ production in presence of mentioned cytokines.

To confirm presence of immune-reactivity in potential CD patients, recent evidences have identified signs of immunological activation in small intestinal mucosa of potential celiac (50). The Authors found that IFN-γ-RNA expression is correlated to intestinal lesions, since it was found lower in potential children than in overt celiac. They hypothesized

that regulatory mechanisms prevent the progression toward a complete mucosal damage. Furthermore they found levels of IL-10 RNA expression higher in potential with Marsh 0 than potential with Marsh 1 lesions, confirming the involvement of IL-10 in controlling of inflammatory responses (50).

In potential CD low grade of inflammation may be related to protective regulatory system that prevents progression toward intestinal damage. In agreement with studies suggesting presence of Treg cells in CD, in our study we identified a heterogeneous panel of responses to gliadin and gliadin peptides, but generally the IFN-γ-production seems to be increased by neutralizing protective effect of Treg.

Interestingly we found by comparative analysis of IFN-γ secretion between potential, celiac (adult and children) patients, and non celiac controls that a significantly different responses among potential and non celiac controls (this data is confirmed when compared celiac and non celiac) was found. This data could provide a clinical support in cases which diagnosis is difficult.

Remarkable, our results reported that T cell lines reactive to three immuno epitopes of α-, ω- and γ-gliadin were found in celiac childen, despite a large heterogenety in set of responses. Furtermore, we have shown that potential have similary pattern of responses to celiacs, with absence of a predominant immunodominance from one or plus peptides.

Previously Vader and coworkers identified a panel of novel epitopes recognized from celiac children $DQ2^{+}$. For these patients a large heterogeneity in the specificity of T cells was observed. No strong immunodominance was found, since the responses were directed to different set of peptides (8).

Overall these data highlighted a great hetereogenus immunogenicity and suggest that probably several epitopes were involved in onset and development of celiac disease.

In the last part of this study, we focussed our attention on role of deamidation in celiac disease. In our population immune-reactivity to naïve and deamidated gliadin, in both potential and overt celiac, was found. Overall we identified a positive reactivity to deamidated gliadin in (~70%) celiac and (~50%) and potential. Surprisingly similar

reactivity to gliadin in naïve form were identified in celiac (50%), while lower responses (~38%) were found in potential celiac. In potential CD role of neutralizing antibodies seems to be less relevant. However may not be true, because due to technical issues not all responders to naïve gliadin were assayed in presence of neutralizing antibodies. Generally we confirmed that potential and celiac children have shown a response to naïve and deamidated gliadin with increasing responses in presence of neutralizing antibodies.

According to several studies, T cell lines and clones isolated from adult celiacs patients were specifically activated by deamidated gluten peptides (15). Specific reactivity to naïve variants were not or only faintly found. First in 2002 Vader and co-workers have analyzed naïve and deamidated gluten cells reactivity in T cell lines generated from pediatric celiacs. Results highlighted a great heterogeneity in intestinal T cell responses, with a number of patients responsive to deamidated and/or to naive form of gliadin. Furthermore specific response to some identified peptides did not required deamidation to be active. Basing on these features, the Authors hypothesized that in celiac patients T cell

response could start toward non deamidated gliadin peptides, and subsequently be driven toward deamidated epitopes. Recognition of few native peptides could trigger the IFN-γ production responsible of a positive inflammatory loop and of consequent low-grade inflammation that lead to tissue damage and to TG2 release (8). The result is that the $CD4^+$T cell response is strongly enhanced with more IFN-γ production, with tissue damage, and increased release of TG2. Alternatively infections may occur in the gastrointestinal tract and activate the loss of tolerance to naive gluten peptides and activation of TG2.

In accordance with this hypothesis our data show reactivity to gliadin and to five immune-dominant gliadin peptides assayed. We identified a large heterogeneous set of responses. Surprising one patient (CD13) failed to recognize gliadin, but showed reactivity to naive DQ2.5-glia-α1a, -α2 and DQ2.5-glia-ω-1,-ω-2, and to DQ2.5-glia-γ-1 γ-gliadin epitopes.

Overall these data could be useful to define the repertoire of gluten peptides relevant for CD pathogenesis and their immune reactivity. The identification of bioactive gluten peptides, involved in CD, could be used

for the engineering of a peptide-based therapy (31). To date a large number of gluten peptides are identified for their ability to stimulate T-cell responses. Several studies on Norwegian and Scandinavian subjects converged in finding a genic region encompassing 33-mer peptide of α-gliadin highly T-cell stimulatory capacity (15,18). Contrasting results were obtained from a Netherland group showed that only 50% of intestinal T-cell lines from adult CD patients were reactive to epitopes of N-terminal region of alpha-gliadin (8). Two immunodominant peptides corresponded to the α-glia-17mer and the DQ2-ω-1 found highly reactive in Italian CD cohort. Recently a large screening (9), have allowed the identification of several epitopes derived from gliadin and related prolamines (19). Collectively, these studies have formed the basis for novel diagnostics and therapies, some of which are currently under clinical investigation.

To data it is indeterminate the number of gluten immunogenic peptides. Despite the large number of immunogenic peptides identified until now, the reduced diversity in T cell responders, and the high redundancy in

gluten peptides recognised open the doors to new peptide based immunotherapy.

Up to now it has been shown that the repertoire of antigens involved in the beginning of the immune response to gluten, and in active CD and in potential CD, could be different from those found in adults CD subjects. Our results confirmed that children may present a high specificity to gluten peptides found immunodominant in adult celiac patients.

In conclusion, our data provided that in potential CD there are evidences of inflammatory pathway, despite the absence of intestinal damage.

To improve our knowledge, potential celiac disease represents an important system to understand the pathways that are able to induce the intestinal damage in genetically predisposed individuals, and clarified the events necessary to onset of the disease.

Could be interesting further to explore factors involved in first stages, and then in progression of mucosal damage.

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Review

Cereal-Based Gluten-Free Food: How to Reconcile Nutritional and Technological Properties of Wheat Proteins with Safety for Celiac Disease Patients

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Abstract: The gluten-free diet is, to date, the only efficacious treatment for patients with Celiac Disease. In recent years, the impressive rise of Celiac Disease incidence, dramatically prompted changes in the dietary habit of an increasingly large population, with a rise in demand of gluten-free products. The formulation of gluten-free bakery products presents a formidable challenge to cereal technologists. As wheat gluten contributes to the formation of a strong protein network, that confers visco-elasticity to the dough and allows the wheat flour to be processed into a wide range of products, the preparation of cereal-based gluten-free products is a somehow difficult process. This review focuses on nutritional and technological quality of products made with gluten-free cereals available on the market. The possibility of using flour from naturally low toxic ancient wheat species or detoxified wheat for the diet of celiacs is also discussed.

Keywords: Celiac Disease; cereals; wheat gluten; dough; immune toxicity

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1. Introduction

In the past 100 years, the human being has experienced a biological transformation with a speed greater than the trend of the last millennia. Over the past seventy years, the diet of Western man has been totally revolutionized: farm food, once characterized for being fresh and free of toxin residues, additives and preservatives, has been almost completely replaced by food products of an industrial chain that is, in the great majority of cases, oriented towards profit and the creation of induced needs. All this has radically changed, and still continues to change, the biological systems in humans leading to metabolic syndrome, allergies and intolerances, among both children and adults, in developed and developing countries.

In this context, in the last two decades, a series of epidemiologic studies have shown a particular increase in Celiac Disease (CD), a life-long intolerance to gluten proteins (the seed storage proteins) present in most cereals [1], both in the United States and Europe, and in developing countries [2,3]. In these subjects, the consumption of cereals containing gluten causes a chronic inflammatory process leading to lesions in the small intestine and a dysfunction in nutrient absorption [1]. The CD treatment, therefore, is based on a strict gluten-free diet throughout the patient's lifetime. Though this dietary regimen guarantees the full recovery of small intestine architecture and functions, for many patients it is strongly restrictive, especially for social events and during travelling. In addition, it is a great task for the food industry to provide safe food that partly resembles in taste and appearance pasta, bread and other baked goods. Furthermore, the nutritional importance of wheat proteins should not be underestimated, particularly in less developed countries where bread, noodles and other products (e.g., bulgur, couscous) may represent substantial diet components.

2. Celiac Disease

Celiac disease is the most common food induced enteropathy in humans. CD is strongly associated with particular HLA genotypes, as only individuals carrying the DQA1*0501 and DQB1*0201 (DQ2), or DQA1*0301 and DQB1*0302 (DQ8) alleles develop the disease. Gluten intolerance presents a large variety of symptoms including gastrointestinal and extra-intestinal manifestations, though in some patients, particularly of pediatric age, the disease is completely symptomless [4,5]. Typical clinical manifestations of CD include chronic diarrhea, weight loss and anemia, mainly caused by malabsorption, as a direct consequence of intestinal villous atrophy. However, a significant proportion of patients have "atypical" form, characterized by extra-digestive symptoms, including skin lesions, isolated hypertransaminasemia, bone pains and fractures, and infertility [6]. Several serological screening detecting the CD-associated anti-tissue transglutaminase (or anti-endomysium) IgA antibodies, and followed by endoscopy, have shown that approximately 1% of the general population have celiac disease [7,8]. However, Maki and co-workers found that the total prevalence of CD is even higher, reaching 1.99% in the Finnish population [9]. The wide spectrum of clinical manifestations, together with the absence of clear symptoms in some cases, could explain why the prevalence of CD is so underestimated. Regarding the pathogenic mechanisms, it is widely accepted that CD is an immune mediated disorder, in which intestinal CD4+ T cells, highly reactive to dietary gluten, have a pivotal role in disease pathogenesis [10]. In addition, recent studies have indicated the prominent role of both

innate immune cells and adaptive CD8+ T cells in damaging the mucosal tissue [10,11]. To date, the only treatment for celiac disease patients is the lifelong complete exclusion of the gluten from the diet. With such a dietary restriction, clinical symptoms, serological markers, and duodenal mucosa histology normalize [12], however gluten reactive T cells remain in the small intestine of patients on gluten-free diet, and re-exposure to the gluten results in immune activation and in mucosal damage. As a consequence, gluten avoidance is strongly recommended; nevertheless, dietary compliance is imperfect in a large number of patients for several reasons, including the need of flavor and palatability of gluten-free cereal-based food [13].

3. Gluten, the Dough "Treasure"

Gluten is one of the earliest protein fractions described by chemists, (a first description by Beccari was in 1728), and it is defined as the "cohesive, visco-elastic proteinaceous material" that remains when wheat dough is washed to remove starch granules and water soluble constituents [14,15]. Gluten is formed by storage proteins necessary for plant germination [16]. In these respects, the wheat storage proteins may not differ much from those of other cereals [14,17], however, the distinctive feature that makes wheat unique is, precisely, the visco-elasticity of gluten. When the grain is milled and mixed with water, storage proteins form a dough, capable of retaining gas bubbles. These properties make wheat suitable for the preparation of a great diversity of food products, including breads, noodles, pasta, and cookies, and it has been the subject of extensive attention by the food industry. Of note, in order to meet the consumer needs, food industries have made in the last fifty years an indiscriminate use of wheat gluten even in food that is naturally devoid, as sauces, chips, cold cuts.

Gluten contains hundreds of proteins, which are present either as monomers or as oligo- and polymers, linked by inter-chain disulphide bonds [15,17], and characterized by high contents of glutamine and proline (namely prolamins), and by low contents of charged amino acids. The new system of classification, based on the availability of complete amino acid sequences, divided the gluten proteins in three broad groups: sulphur-rich (S-rich), sulphur-poor (S-poor), and high molecular weight (HMW) prolamins ([18], Figure 1). Traditionally, gluten proteins have been divided according to their solubility in alcohol-water solutions (e.g., 60% ethanol), as the soluble gliadins and the insoluble glutenins [19]. These properties are largely determined by the inter-chain disulphide bonds, with the glutenins consisting of disulphide-stabilized polymers. Reduction of these inter-chain bonds allows the separation of the glutenin subunits into low molecular weight (LMW) and high molecular weight (HMW) groups (Figure 2). In contrast, the alcohol-soluble gliadin fraction consists mainly of monomeric proteins, which either lack cysteine (ω-gliadins), or have only intra-chain disulphide bonds (α-type and γ-type gliadins) (Figure 2). However, small amounts of polymers related to the glutenins are also present in the gliadin fraction. These appear to differ from the alcohol-insoluble glutenins, as they have lower M_rs and higher contents of LMW subunits [20–22].

Figure 1. Schematic representation of types and peculiarities of wheat gluten proteins (gliadins and glutenins-GS; modified from [17]).

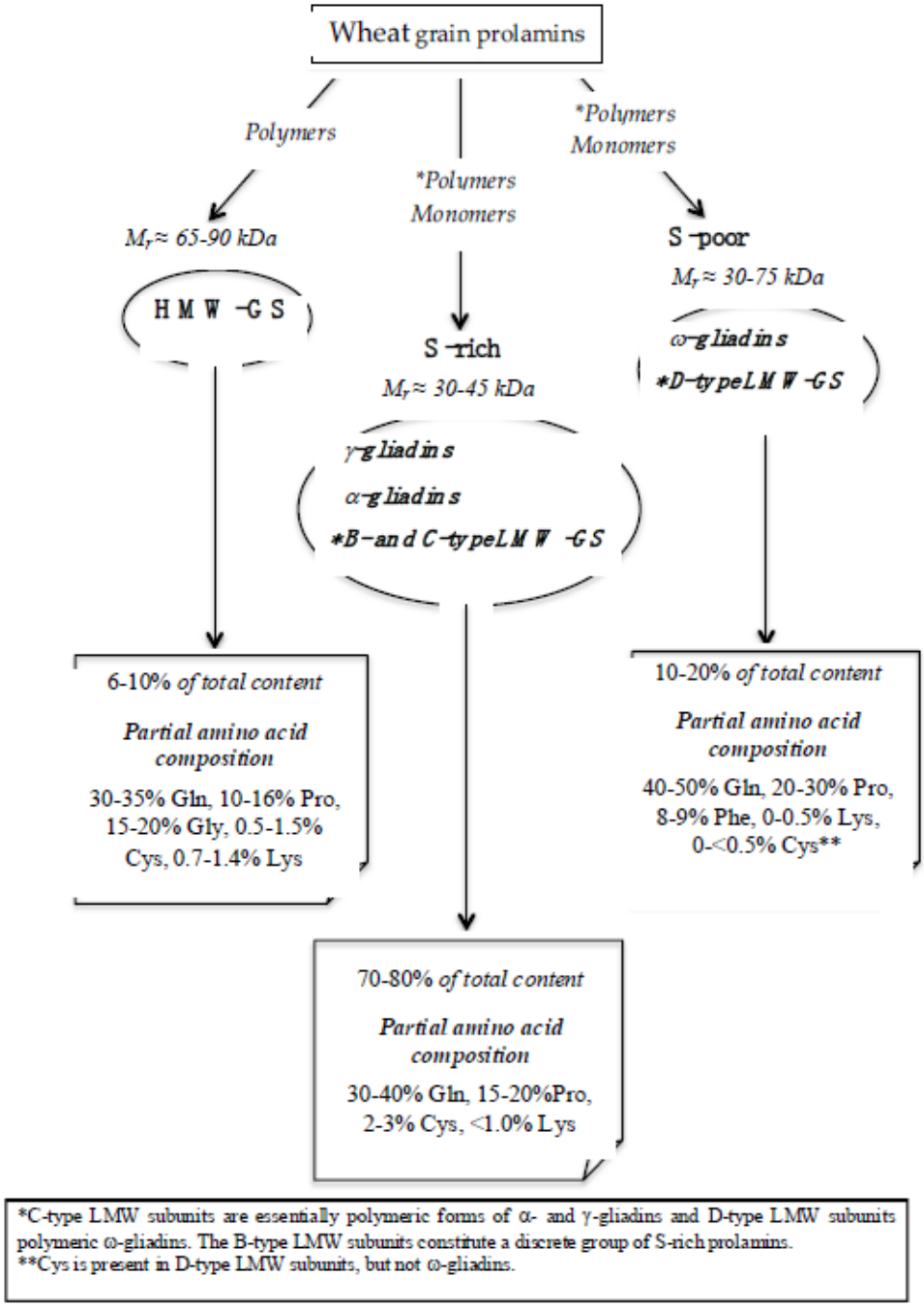
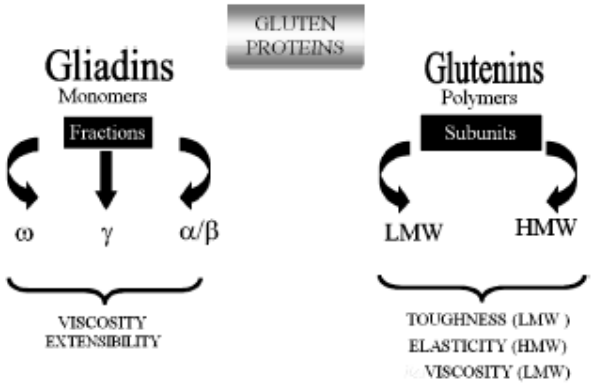
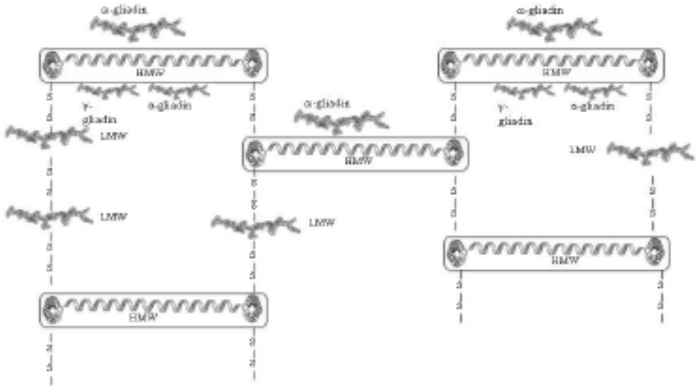


Figure 2. Gluten proteins: fractions and technological properties [23].



Both fractions are important contributors to the rheological properties of dough (Figure 2), though their functions are divergent. Hydrated gliadins have little elasticity and are less cohesive than glutenins, and contribute mainly to the viscosity and extensibility of the dough. In contrast, hydrated glutenins are both cohesive and elastic and are responsible for dough strength and elasticity [24,25]. A proper mixture of both fractions is essential for the quality of the end product. However, of particular importance are the glutenin polymers, and it is well established that strong (*i.e.*, highly visco-elastic) doughs contain high proportions of HMW glutenin polymers [24]. Numerous studies are consistent with the hypothesis that the HMW subunits form an elastomeric polymer network which provides a "backbone" for interactions with other glutenin subunits and with gliadins (Figure 3) [26,27]. There is no doubt that this network is mainly stabilized by inter-chain disulphide bonds [28,29]. Additional covalent bonds formed during dough making are tyrosine-tyrosine and thiyl-tyrosine crosslinks between gluten proteins [30–32]. However, the covalent structure of the gluten network is superimposed by non-covalent bonds (hydrogen bonds, ionic bonds, hydrophobic bonds) [33].

Figure 3. A structural model for wheat gluten in which the HMW subunits provide a disulphide-bonded backbone that interacts with other gluten proteins by disulphide bonds (LMW subunits) and non-covalent interactions (gliadins) (modified from [26]).



4. Formulation and Nutritional Value of Gluten-free Products

The food not allowed in the gluten free diet include: (a) all types of bread and food prepared with wheat flour, including kamut and spelt, rye, barley, triticale, or with ingredients from these flours; (b) food that contains wheat, or derivatives of gluten used as thickeners, such as hot dogs, salad dressing, sauces, canned, some types of cheese and cold cuts; (c) medicinal products that use gluten as binder in the pills or tablets.

Though from a nutritional point of view, gluten exclusion does not entail particular problems, being a mixture of proteins with low nutritional and biological value, the gluten-free diet creates enormous limitations, above all in the social activities related to food. In addition, this dietary therapy has, often low content of vitamins and ions, such as vitamins B and D, calcium, iron, zinc, and magnesium, as well as fiber [34–36]. Furthermore, one of the major risks is the risk to develop obesity and diseases related to metabolic syndrome [37]. However, the high technological value renders gluten almost indispensable in baked products, and its replacement, as structure-building protein, presents a major technological challenge for the food industry. Although many advances have been made in the preparation processes of gluten-free products, using starches, hydrocolloids, gums and novel ingredients [38], many gluten-free industrial products available on the market exhibit a low nutritional quality, poor mouth feel or flavor [39] and, no less important, are particularly expensive. As a consequence, the research interest to develop gluten-free products has significantly increased in recent years. The pilot study by Rotsch [40] showed that bread could be prepared from starch and gel-forming substances, since starch, combined with water, at a temperature included between 60 and 80 °C, increases in volume, in a sort of swelling (a process called gelatinization) [41–43]. This process increases the product consistency by mimicking the gluten viscoelastic properties. Thickeners and gums/hydrocolloids, derived from various seeds, fruits, or plant extracts, could be also added in these formulations to improve water retention, texture and appearance properties [44–47]. Among different types of flours, present on the market and rich in starch, rice and corn flour are the best suited to the gelatinization process. These starches, are polysaccharides composed of amylose and amylopectin, present in the original product in the percentages equal to 20%–30% and 70%–80%, respectively.

The amylose is a linear polymer of glucose in which the glucose units are held together by α (1→4) bonds, whilst the amylopectin is a branched polymer of glucose, similar to amylose, structured with side chains that are grafted every 24–30 glucose units through α (1→6) bonds. Amylopectin is the most responsible for the gelatinization of starch granules in the presence of water and heat. During this process a fraction of both amylopectin and amylose goes into solution, however, the starch gelatinization is inversely proportional to the amylose content [48,49]. From a nutritional perspective, the more starch is gelatinized (because of its reduced amount of amylose), the more it is hydrolysable by alpha-amylase, with an increase of the glycemic index [50,51]. In contrast, with a greater amylose content, the gelatinization process is reduced, as well as the glycemic index. Gluten-free food prepared with corn and rice starch have a high glycemic index [52,53] and increase the risk to develop metabolic syndromes in celiac patients [54,55]. In fact, it is well known from epidemiological studies that the daily consumption of high glycemic index food is correlated with the risk to develop cardiovascular disease, obesity and diabetes [56–60]. Furthermore, the use of palm oil, cream powder,

microencapsulated high-fat powder, and low-fat dairy powders [39,40], aimed to improve the palatability of cereal-based gluten-free products, renders these products highly caloric.

5. Recent Advances in Formulation of Cereal-Based Gluten-Free Food

In addition to the high glycemic index and caloric density of cereal-based gluten-free food, these products generally are not enriched/fortified, so that, they may not contain the same levels of nutrients, as the natural wheat grains. Therefore, uncertainty still exists whether celiac patients compliant with gluten-free therapy have a nutritionally balanced diet. Grehn *et al*. screened the intake of nutrients and foods of 49 adults diagnosed with coeliac disease and following a strict gluten-free diet [61]. They had a lower intake of fibre when compared to a control group on a normal diet. In their studies with coeliac adolescents, Mariani *et al*. [62] concluded that adherence to a gluten-free diet worsens the nutritionally unbalanced diet in adolescents, as it has been thereafter confirmed by Thompson *et al*. [63]. For these reasons several studies investigated the preparation of a new generation of cereal-based gluten-free food. The enrichment of baked products with dietary fibres and devoid of gluten has been the goal of various technologist teams. Gallagher *et al*. incorporated inulin (8% inclusion level) into a wheat starch-based gluten-free formulation [64]. Inulin is a non-digestible polysaccharide that is classified as a dietary fibre. It also acts as a prebiotic by stimulating the growth of "healthy" bacteria in the colon [65]. When added to wheat bread, inulin improves loaf volume, increases dough stability and produces a uniform, and finely grained, crumb texture [66].

Gambus *et al*. replaced corn starch with amaranth flour to enhance the protein and fibre contents of gluten-free breads [67]. At a 10% replacement level, protein and fibre levels increased by 32% and 152% respectively, whilst sensory quality was unaffected. Taylor and Parker discussed the use of quinoa in the production of enriched gluten-free bakery goods [68]. Both quinoa and amaranth are *dicotyledonous* species that are not related to the actual cereals (*monocotyledons*), such as wheat, barley and rice. They are also called pseudocereals, since these species produce small seeds that resemble those of cereals. The grain of pseudocereals does not contain gluten proteins but it is rich in proteins with high biological value (albumins and globulins) and contains carbohydrates that can be considered nutraceuticals, as they have cholesterol- [69,70] and glycemic-lowering effects, and induce a reduction of free fatty acids [52]. Importantly, the total absence of immune toxicity of amaranth storage protein for celiacs patients has been demonstrated [71], and several studies were carried out in order to improve the structural properties of quinoa and amaranth as ingredients for bread, pasta and crackers [72–74].

6. Nutritional Quality of Wheat Flour: Advantages in Introducing a Cereal-Based Gluten-Free Food in Diet of Celiacs

The therapy with gluten-free products, besides the risk of nutrient deficiency and metabolic syndrome, as described above, entails the difficulty of maintaining the cure over time. Reduced palatability and taste of gluten-free food create enormous limitations in the diet of patients. To solve these issues, numerous studies are currently devoted to the use of *in vitro* detoxified flour or flour from ancient wheat cultivars, in the formulation of pasta and baked goods.

6.1. Nutritional and Health Properties of Hexaploid and Tetraploid Wheat

Worldwide, the number of people who eat wheat for a substantial part of their diet reaches several billions. Because of the high content of starch, (about 60%–70% of the whole grain and 65%–75% of white flour) wheat is often considered no more than a source of calories. Despite its relatively low protein content (usually 8%–15%), wheat still provides as much protein for human and livestock nutrition as the total soybean crop (as calculated in reference [75]. However, the lysine content of wheat is low and varies significantly from grain to flour [15]. Grain of high protein content has very low content of lysine approximately 30 mg g^{-1} protein [76]. Wheat is a source of minerals such as Zn (20–30 mg Kg^{-1}) and Fe (30–36 mg Kg^{-1}), contributing to 44% of the daily intake of iron (15% in bread), and 25% of the daily intake of zinc (11% in bread) in the UK [77] Wheat is also a source of selenium which varies widely from about 10 µg Kg^{-1} to over 2000 µg Kg^{-1} (FAO/WHO, 2001; [78]). The concentration of selenium in wheat is largely determined by the availability of this element in the soil. Wheat produced in Western Europe may contain only one-tenth of the selenium that is present in wheat grown in North America. Wheat also contains a range of components with established health benefits that are concentrated, or solely located, in the bran. In addition, the following components are either present in low amounts, or completely absent, and with a large variation in their concentrations:

- **lignan**s, a group of polyphenols with phytoestrogen-like activity, present at levels up to 10 µg g^{-1} in wholemeal wheat and almost 20 µg g^{-1} in the bran [79];
- **phenolic acid**s that in wholemeal range up to almost 1200 µg g^{-1} [80]. They represent, quantitatively, the major group of phytochemicals in the wheat grain and are derivatives of either hydroxibenzoic acid or hydroxycinnamic acid. Epidemiological studies indicate that phenolic acids have a number of health benefits which may relate to their antioxidant activity; furthermore, a high correlation between the total antioxidant activities of grain and their phenolic acid contents has been reported [81,82];
- **folate**s that in wholemeal varied from 364 to 774 ng g^{-1} dry weight in winter wheats and from 323 to 741 ng g^{-1} dry weight in spring wheats, and positively correlated with the bran yield [83];
- **dietary fibre** derives from polymers of wheat endosperm cell wall: they are constituted mainly by arabinoxylans (approximately 70%) and (1-3) (1-4)β-$_D$-glucans (approximately 20%). The arabinoxylans are present in both soluble and insoluble forms, being the former considered to have health benefits [84,85]. However, insoluble fibre may also favour the delivering phenolic antioxidants into the colon, with a reduction of colon-rectal cancer risk [86]. Gebruers *et al*. showed wide variation in the contents of total and water-extractable arabinoxylans in both white flour and bran fractions [87]. Similarly Ordaz-Ortiz *et al*. showed variation from 0.26% to 0.75% dry weight in the content of water-extractable arabinoxylan in 20 French wheat cultivars and from 1.66% to 2.87% dry weight in total arabinoxylans [88].

6.2. Ancient Wheats

Triticum monococcum, was the first wheat to be cultivated by man and is a diploid species characterized by the presence of the AA genome. Due to the simplicity of its genome, *Triticum monococcum* has

attracted the interest of the scientific community on nutritional and health aspects in relation to celiac disease. If we consider, in fact, that for each genome (AA, BB and DD) there are dozens of genes coding for prolamins in wheat caryopses, it is evident that in *Triticum monococcum* the mere presence of the AA genome encodes for a reduced variety of gluten proteins (and of potential immune toxic peptides). Recent evidence shows that prolamins of some *Triticum monococcum* cause a reduced inflammatory effect in celiac patients [89–92], particularly for the inability to activate the innate branch of immune cells [89], or to induce apoptosis of enterocytes [93]. The reduced, or absent, toxicity of *Triticum monococcum* prolamins can then be interpreted in two ways, namely via (*i*) a low presence of toxic peptides; (*ii*) an abundant presence of protective sequences. However, other *in vitro* and *in vivo* studies have warned about the safety of some monococcum cultivars for celiac patients [94,95], thus suggesting that the immune toxicity for celiacs might strictly depend on the specific *Triticum monococcum* varieties.

The search of naturally detoxified, or less toxic, ancient grains is of great interest for their potential use in the general diet to prevent disease in those individuals at high risk to develop gluten intolerance.

Regarding the technological properties, though some studies have considered *Triticum monococcum* not suitable for bread and pasta production [96,97], it has been demonstrated that the aptitude to bread-making is extremely different and depends on the specific varieties analyzed [98,99]. Studies carried out at an industrial level have shown that pasta made with 100% of *Triticum monococcum* flour has very low loss of starch during cooking comparable to that made from durum wheat semolina [23]. These findings on the bread- and pasta-making aptitude of *Triticum monococcum* flours are encouraging, and indicate a real chance to select for ancient varieties with superior technological characteristics and reduced toxicity for celiacs.

6.3. Detoxification of Wheat Gluten

Numerous studies are currently devoted to prepare pasta and baked goods made from wheat flours modified in order to eliminate, or reduce, the immune toxicity of gluten proteins (detoxification process).

A first method, using endopeptidase of bacterial origin during the preparation of wheat flour dough, results in the complete degradation of gluten peptides including those that are strongly immune toxic for celiacs [100]. Such an approach, carrying out a total destruction of the gluten network, reduces the technological properties (viscoelasticity) of dough and, consequently, of pasta or baked goods, unless the flour is integrated with structuring agents, as pre-gelatinized starch, emulsifiers or hydrocolloids.

Another method to detoxify gluten proteins uses the specific transdamidation of toxic epitopes done by the tissue-transglutaminase of microbial origin (*Streptomyces mobaraensis)* in the presence of lysine methyl ester [101]. This method has the great advantage of blocking the immunogenicity of T cell epitopes (as demonstrated in an *in vitro* assays using intestinal T cell from celiacs), and more importantly, it keeps intact the gluten network and preserves the technological properties of the flour. Furthermore, this procedure uses an enzyme largely employed in the food industry for improving the texture of foods. A preliminary 90-days trial made with CD subjects in remission consuming bread slices with transamidated gluten indicated that only a subgroup of celiacs exhibited clinical symptoms compared to subjects consuming the toxic gluten [102]. The researchers have now implemented the

transamidation reaction in order to reach protection in the great majority of CD volunteers that eat detoxified wheat flour.

7. Conclusions

The gluten proteins contained in wheat flour are crucial during the bread and pasta making process, since they confers to the dough its viscosity and elasticity. In addition, wheat flour provides to foodstuffs important nutritional components, including dietary fibre, vitamins, and minerals. These properties account for wheat being cultivated by man in such enormous quantities throughout the world. However, gluten proteins are also responsible for a very common, and in most cases very severe, intolerance in a large number of individuals. To date the only safe and efficacious therapy for people with celiac disease is the long-life avoidance of gluten from the diet.

The replacement of the unique technological properties of wheat gluten represents the major task of industry for providing high quality gluten-free foods, such as pasta, bread and baked products, in terms of structure, loss of starch during cooking, and optimal cooking time.

Nutritional studies on people with CD on a gluten-free diet revealed several nutrient deficiencies, particularly of vitamins and minerals, as well as an increased of obesity risk, this latter due to the high glycemic index of the gluten-free diet. However, despite the numerous studies, it has not yet been possible to offer celiac patients an alternative diet therapy, based on high nutritional and tasty cereals that are naturally gluten-free. Foodstuffs made with technologically detoxified wheat flour, or with wheat varieties with naturally low content of toxic gluten sequences, or more suitable to gastrointestinal enzymatic degradation, would be a much-desired dietary therapeutic alternative. An extensive production of foodstuffs made with detoxified wheat flour that could be commonly consumed not only by those people suffering gluten intolerance, but also by the rest of the population could be envisaged; the large diffusion of such products could have the goal, in a totally innovative way, of reducing the immune sensitization to gluten and, likely, decreasing the incidence of celiac disease.

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Conflicts of Interest

The authors declare no conflict of interest.

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