Proceedings of the Nutrition Society (2010), **69**, 347–353 doi:10.1017/S0029665110001825

The 3rd International Immunonutrition Workshop was held at Platja D'Aro, Girona, Spain on 21–24 October 2009

3rd International Immunonutrition Workshop

Session 4: Dietary strategies to prevent and mitigate inflammatory diseases
Dietary strategies of immunomodulation in infants at risk for celiac disease

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Celiac disease is an inflammatory disorder of the small intestine, triggered by the ingestion of gluten proteins contained in wheat, barley or rye, in genetically susceptible individuals. This disorder is considered to be mainly mediated by cellular immunity and restricted to the human leucocyte antigen-DQ presentation of gluten-derived toxic peptides to T-cells. Moreover, the involvement of innate immunity has been recently demonstrated to be necessary also for the development of intestinal tissue damage. Genetic susceptibility accounts for an uncertain proportion of the disease risk and gluten introduction works as the precipitating factor. However, currently, the research interest is also focused on environmental factors and gene–environment interactions, especially during the first months of life, which might help explain the onset of the disease. Infectious and dietary factors that could modulate the immune response orientating it either towards tolerance or intolerance/autoimmunity are the focus of primary attention. A significant number of studies have looked into the protective effect of breast-feeding against the disease. It is generally accepted that breast-feeding during the introduction of dietary gluten and increasing the duration of breast-feeding are associated with reduced risk of developing celiac disease. However, it is still not fully established whether breast-feeding truly protects with permanent tolerance acquisition or only reduces the symptoms and delays the diagnosis. Moreover, the timing and dose of gluten introduction also seem to be relevant and long-term prospective cohort studies are being carried out in order to elucidate its role in celiac disease development.

Celiac disease: Disease risk and prevention: Breast feeding: Gluten introduction: Tolerance to gluten: Probiotics

Celiac disease (CD) is an inflammatory disorder affecting primarily the small intestine and triggered by the ingestion of gluten proteins contained in wheat, barley or rye, in genetically susceptible individuals. It is generally accepted that CD is a T-cell-mediated disease, in which gliadin-derived peptides bound to human leucocyte antigen (HLA)-DQ2 (DQA1*05/DQB1*02)/DQ8 (DQA1*0301/DQB1*0302) molecules activate lamina propria infiltrating T-lymphocytes. The subsequent release of pro-inflammatory cytokines, in particular interferon (IFN)-γ, leads to a profound tissue remodelling, flattening of the small intestinal mucosa and nutrient malabsorption. However, currently the role of the innate immune response is gaining extraordinary importance, since it seems to be involved in several ways in the pathogenesis of the disease. Thus, it has been suggested that innate immunity collaborates with adaptive immunity to

Abbreviations: APC, antigen presenting cells; CD, Celiac disease; CDA, CD autoimmunity; IEL, intraepithelial lymphocytes; IFN, interferon; HLA, human leucocyte antigen; TCR, T-cell receptor; TLR, Toll-like receptors.

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induce a pro-inflammatory T-helper 1 response, to increase the number of intraepithelial lymphocytes (IEL) and to favour the cytolytic attack of the epithelium[1,2]. CD is a complex and multifactorial disorder in which the interplay between environmental and genetic factors determines the aberrant immune response to gluten proteins. The major genetic risk factor involved in CD is represented by HLA-DQ genes. Over 90% of CD patients express HLA-DQ2 or in *trans* position in HLA-DR5/DR7 heterozygous patients. The remaining celiac patients express DQ8[3]. However, only 3–5% subjects expressing DQ2 or DQ8 actually develop CD and on the other hand, over 60% of familial clustering remains unexplained by HLA genes[4], which means that the identification of other genetic loci and environmental risk factors is needed in order to gain new insight into CD pathogenesis.

Wheat gliadins and other prolamines from barley and rye are cereal storage proteins (collectively termed gluten) unusually rich in proline and glutamine. The deamidation of glutamine into negatively charged glutamic acid by the enzyme transglutaminase expressed in the intestinal lamina propria promotes the interaction of gluten-derived peptides with the peptide pocket of HLA-DQ2/DQ8 molecules, which can be recognized by T-cells[5,6]. These gluten-specific CD4+ T-cell clones produce IFN-γ[7]. However, studies in animals have shown that mice engineered to express human CD4 and the HLA-DR3-DQ2 haplotype[8] or human CD4 and HLA-DQ8[9] and primed with gliadin generate peptide-specific T-cell clones that respond to gluten dietary exposure, but this response does not lead to the development of enteropathy. Other transgenic mice, expressing the human DQ8 heterodimer and mucosally immunized with gliadin and cholera toxin as adjuvant, were simultaneously treated intragastrically with *Lactobacillus casei* ATCC 9595. The co-administration of *L. casei* to sensitized mice enhanced the gliadin-specific response mediated by CD4+ T-cells increasing the IFN-γ expression. However, neither the immunization with gliadin and cholera toxin nor the addition of *L. casei* resulted in any morphological alteration of the small intestinal mucosa[10]. These humanized models indicate that gluten ingestion can be tolerated even when CD4+ T-cell immunity to gluten is established, raising the question of the complementary mechanisms that contribute to breaking mucosal tolerance to gluten and turning a controlled immune response into chronic inflammation and epithelial damage.

The role of innate immunity and viral infections in celiac disease development

Interpretations up until the present time point to the interplay between adaptive and innate immune mechanisms orchestrated by IL-15 driving IEL activation and epithelial damage in CD pathogenesis[2]. IEL (CD8+ T-cell receptor (TCR) αβ+ and TCRγδ+) are hallmarks in active CD. While after starting a gluten-free diet numbers of CD8+ TCRαβ+ return to normal in parallel with villous architecture recovery, TCRγδ+ IEL remain high for years[11,12]. These findings led to the view that CD8+ TCRαβ+ exert a deleterious effect on epithelial cells that can be antagonized by TCRγδ+ IEL[12]. CD8+ TCRαβ+ are involved in TCR-independent natural killer-like killing of intestinal epithelial cells through binding of the natural killer group 2D (NKG2D) receptor expressed in CD8+ lymphocytes to its ligand MHC class 1-related chain A (MICA) in epithelial cells[1,13]. Both NKG2D- and MICA-enhanced expression in active CD might be driven by IL-15[13,14]. It has been documented that gluten toxic fragments, different from those proved usual CD4+ T-cell epitopes, induce a rapid production of IL-15 by lamina propria macrophages and dendritic cells[15]. IL-15 has also been shown to stimulate, *ex vivo*, IFN-γ secretion by IEL[16]. IFN-α is another cytokine that has been shown to be up-regulated in the mucosa of active CD[17] and is a known inducer of T-helper 1 responses. Since IFN-α and IL-15 are key players of the early innate responses to intracellular pathogens, the capability of infectious agents to act as a triggering factor in the inmunopathogenic mechanisms leading to the breakage of tolerance to gluten has been considered. Indeed, double-stranded RNA viruses are strong inducers of both cytokines, which might re-instruct or *de novo* recruit and activate dendritic cells to prime gluten-specific T-cells[2]. Prospective, epidemiological evidence supports the role of multiple infections by rotavirus in increasing the risk of subsequent development of celiac autoimmunity in predisposed individuals[18] perhaps through a disruption of the intestinal barrier and facilitation of gliadin epitope penetration. On the other hand, in some studies but not all, analyses of serum antibodies have shown association between past infections with Adenovirus type 12 and Hepatitis C virus and the development of CD. It has also been suggested that molecular mimicry of viral proteins with toxic gluten peptides (homology in amino acid sequences) might modulate the host immune tolerance and trigger the development of CD[19,20].

Possible involvement of Toll-like receptors in the innate and adaptive response in celiac disease

Toll-like receptors (TLR) are a class of highly conserved membrane bound molecules which have a principal role in the recognition of pathogenic and non-pathogenic microorganisms and thus act in the primary line of defence. Dendritic cells, macrophages and epithelial cells, among other cell types, express TLR which recognize microbial products, such as danger signals released from microorganisms, as well as cell wall components, lipoproteins, genome sequences, etc. which through its binding exert a regulation on the activation of innate and adaptive immune responses. The activation of TLR also directly or indirectly influences regulatory T-cell functions[21]. These findings suggest that different TLR with different specificities and the integration of their signals participate both in immune activation and immune regulation. It has been suggested that TLR might possibly be involved in the modulation of immune responses leading to CD. The observation of increased levels of TLR2 and TLR4 mRNA in the mucosa of celiac patients suggests a possible inherent defect in this branch of the innate immunity[22]. Interactions with these receptors in the surface of antigen presenting cells (APC)

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https://doi.org/10.1017/S0029665110001825 Published online by Cambridge University Press

might in fact activate them and lead to the adaptive immune response and also the breakage of self-tolerance and the development of autoimmunity. In fact, self-reactive antibodies from celiac patients have shown the capacity to bind TLR4 and cause monocyte activation[20]; this might also be a consequence of molecular mimicry among harmful proteins (for example from rotavirus) and self-proteins.

Dietary strategies for immunomodulation in celiac disease pathogenesis

The protective role of breast-feeding

A systematic review and meta-analysis of observational retrospective studies published in May 2004, concluded that increased duration of breast-feeding is associated with a reduced risk of CD[23]. Five of the six case–control studies that satisfied the inclusion criteria of methodological quality found that children with CD had been breast-fed for a significantly shorter period compared with controls. Also, the meta-analysis of four of these studies led to the conclusion that the risk of developing CD was significantly reduced in children who were breast-fed at the time of gluten introduction (OR 0·48, 95% CI 0·40, 0·59). However, from the reviewed studies it is not clear whether breast-feeding only delays the onset of symptoms or provides a permanent protection against the disease. On the other hand, the results of the meta-analysis are subject to limitations, such as those derived from a recall bias that might induce misclassification of the duration of breast-feeding and the age of gluten introduction. Moreover, another source of bias might be derived from sub-optimal adjustment for potential confounders across children who were breast-fed and those who were not. For instance, only one of the studies controlled for the HLA genotype, which, notably, was the one study that did not find a relationship between breast-feeding and protection against CD. However, this was a small study with only eight cases of children with CD and it is likely that a type II error has occurred. Given these limitations, it seems clear that long-term prospective cohort studies are required to investigate further the relationship between breast-feeding and CD.

The study by Ivarsson *et al.*[24] is a population-based incident case-referent study of 627 cases with confirmed CD (reported to a CD register between November 1992 and April 1995) and 1254 referents assessing patterns of complementary food introduction to infants. The study revealed that the risk of CD was reduced in Swedish children aged less than 2 years if they were still breast-fed when dietary gluten was introduced (adjusted OR 0·59; 95% CI 0·42, 0·83) and the risk increased when the gluten was introduced in the diet in large amounts (OR 1·5; 95% CI 1·1, 2·1). In the present study, the effect of age at the time of gluten introduction was not conclusive. It is biologically likely that the presence of breast milk at the time gluten is introduced increases the chance of developing oral tolerance for the antigens of importance. It is unclear whether this chance is only effective during a certain period in infancy. In the present study, the exposure risk factors explored were of no or only minor importance in children older than 2 years. Therefore, it is also important to pursue further whether favourable infant dietary patterns postpone the onset of the disease or in fact reduce the overall lifetime risk of the disease[24].

Previous to this analysis of risk factors, the authors had documented an epidemic of symptomatic CD between 1984 and 1996 in Swedish children below 2 years of age, partially explained by changes in infant feeding[25]. The increase in incidence was preceded by an increase in the amount of gluten consumed and by a postponement of dietary gluten introduction which should have resulted in a higher proportion of children introduced to large amounts of gluten after the discontinuation of breast-feeding. Recently, a Swedish study on the prevalence in children born in 1993, during the epidemic and under these unfavourable dietary practices, revealed that the prevalence was as high as 3%, 3-fold higher than the usually suggested prevalence of 1%[26].

As suggested from studies showing the risk reduction of childhood type I diabetes mellitus with breast-feeding and late introduction of cow's milk, it seems plausible that in addition to the immunologically active components in breast milk, avoidance of the early introduction of cow's milk protein contributes to the protective effect of breast-feeding[27]. This might as well be the case in the prevention of CD by breast-feeding. Additionally, differences in the gut microbiota between breast-fed and formula-fed infants might account for the protection observed with breast-feeding against the development of the disease[28].

Timing of gluten introduction

The effect of the timing of gluten introduction was studied by Norris *et al.*[29]. They carried out a prospective observational study from 1994 to 2004 of 1560 children at an increased risk of CD or type I diabetes as defined by possession of either HLA-DR3 or -DR4 alleles or having a first-degree relative with type I diabetes. In these children, they assessed the risk of CD autoimmunity (CDA) defined as being positive for tissue transglutaminase autoantibody in two or more consecutive visits. Infants exposed to gluten in the first 3 months of life have a 5-fold increased risk of autoimmunity compared with infants first exposed at 4–6 months (hazard ratio 5·17, 95% CI 1·44, 18·57). Infants introduced to gluten at 7 months or later also had an increased risk of CDA compared with those exposed between 4 and 6 months (hazard ratio 1·87, 95% CI 0·97, 3·00). This study did not find any evidence for a protective effect of prolonged breast-feeding. The median duration in both CDA positive and CDA negative children was 5 months. This analysis, however, was not restricted to the HLA-DR3 children and possibility exists that the protective effect of breast-feeding was evident if only children with genetic risk were considered. The different findings between this study and others reporting a breast-feeding protective role might be explained by the different methodologies between retrospective and prospective studies and also by the different dietary practices between Europe and the United States[29], since in Europe the introduction of gluten tends to occur as a replacement of breast milk at weaning (for example, the flour-based follow-up infant formula once used in Sweden), whereas in the United States they appear more like two separate events. Some

https://doi.org/10.1017/S0029665110001825 Published online by Cambridge University Press

explanations have been reported by Norris *et al.*[29] to the increased risk of CDA when the first exposure to gluten occurs in younger and older children instead of at the age of 4–6 months. In younger children, this increased risk would be related to the immaturity of the intestinal epithelial barrier and, in this sense, zonulin has been implicated as a protein released in response to gliadin, resulting in further loss of barrier integrity as zonulin acts to disassemble the tight junctions between enterocytes[30]. On the other hand, in children aged 7 months or older, the factor leading to the increased risk of CDA might be the introduction of large amounts of gluten at first exposure[29].

A recent position paper of the ESPGHAN Committee on Nutrition has provided as possible practical suggestions on the introduction of complementary feeding to avoid both early (<4 months) and late (≥ 7 months) introduction of gluten and to gradually introduce small amounts of gluten while the infant is still breast-fed[31]. This change in the policy of complementary feeding is aimed at the modulation of the predisposition of chronic disorders later in life, particularly that of CD. This is, however, a matter of debate, since exclusive breast-feeding for around 6 months is considered a desirable goal both by ESPGHAN[32] and WHO in order to support healthy growth and development and reduce the risk of infections. As suggested by Agostoni & Shamir[27] perhaps the 6-month theorem should be partly revised and small amounts of solids, including gluten, be allowed in the 4–7-month temporary window to modulate the genetic predisposition towards an autoimmune response, especially in developed countries where the exposure risk to infectious agents is different from that in the developing countries.

Is it possible to induce the acquisition of tolerance to gluten?

Administration of antigen by the oral route induces hyporesponsiveness to subsequent challenge with the antigen given in an immunogenic form, usually by a parenteral route, a phenomenon termed oral tolerance[33]. Oral tolerance, however, usually affects the response of the local immune system at the intestinal mucosa, thus, preventing hypersensitivity reactions to food proteins that could lead to disorders such as CD or food allergies[34]. Similarly, immunological tolerance prevents the aberrant immune responses to commensal bacteria in the gut[33]. However, the acquisition of oral tolerance is a complex process and is far from being fully elucidated. Works published in the 1980–1990s led to the idea that the mechanisms responsible for oral tolerance depended on the feeding regime used, inducing tolerance leading either to clonal anergy (or deletion) of specific T-cells or to the induction of regulatory T-cell activity[35]. More recent knowledge has pointed at APC as fundamental players directing tolerance or immunity towards specific antigens. It is the level of expression of co-stimulatory molecules in APC, such as CD80, CD86 or CD40 and the balance between IL-12 and IL-10 produced by APC that seems to determine whether an antigen induces tolerance of productive immunity when presented to a CD4 T-cell[36,37]. The expression of co-stimulatory molecules in APC is controlled by the presence of danger signals from pathogens or even by conserved structures from any kind of microbe that are recognized through their pattern recognition receptors, such as TLR. Finally, current evidence suggests that tolerance requires migration of dendritic cells that have taken up an antigen in the mucosal lamina propria to the mesenteric lymph nodes[38].

Several strategies aimed at down-regulation of pathogenic T-cells by induction of Ag-specific hyporesponsiveness have been assayed to prevent experimental autoimmune diseases. The generation of immunological tolerance has been attempted through ingested or inhaled soluble proteins by the oral and the nasal routes[39]. An attempt to re-induce tolerance to gliadin has been carried out in HLA-DQ8 transgenic mice immunized by intrafootpad injections of gliadin in Freund's adjuvant after they had been previously instilled into the nostrils with soluble gliadin following a tolerization protocol. A decrease in systemic T-cell responses to the recombinant α-gliadin was found as reflected by a lymphocyte proliferation assay. While the immunization protocol induced the transcription of both T-helper 1 and T-helper 2 cytokines, the tolerization protocol down-regulated significantly only the IFN-γ mRNA expression[40]. This finding underlines the potential usefulness of this strategy for the immunomodulation of this disease. However, as we have described above, the presence of gliadin-specific T-cell clones in transgenic mice models is not sufficient to develop enteropathy, which makes the down-regulation of these clones not so relevant without addressing the rest of the pathogenic pathways involved in CD.

Primary prevention in infants at risk for celiac disease through dietary intervention strategies

Exploring the options of primary prevention requires combined epidemiological, clinical and basic scientific research efforts to shed light on the potential impact of life-style factors, genetic determinants, immunological pathways and gene–environment interactions in the development of CD[28]. Some of the most important issues that need investigation in CD in the coming years were identified in 2007 by the European platform on CD (CDEUSSA)[28]. Regarding prevention, their report listed as important issues: (1) to determine the long-term effects of breast-feeding and the molecular basis for the protective effect and (2) to determine the role of timing and dose of gluten during introduction. In addition, the FISPGHAN working group on CD added also the exploration of the role of probiotics and prebiotics in oral tolerance[41].

In line with these priorities, several population studies are being currently carried out to search into new strategies for CD prevention during the first stages of life. PREVENTCD is a project funded under the European Union's Framework Programme 6th which is being performed by 10 European countries in cooperation with the Association of European Coeliac Societies. The project studies the possibilities of induction of gluten tolerance in genetically predisposed children. It is a prospective, randomized, blind dietary intervention study in young children from high-risk

https://doi.org/10.1017/S0029665110001825 Published online by Cambridge University Press

families for CD (http://www.preventceliacdisease.com). The idea is that introducing small amounts of gluten and gradually increasing them during a certain window of opportunity in the infant's development and while being still breast-fed will induce oral tolerance to gluten. A total of 1000 children with a first-degree family member suffering from CD will participate in this intervention study and they will be followed during 3 years. From the fourth to sixth month of life, they will receive a small amount of gluten that will gradually be increased during the sixth to ninth month, and followed by free intake thereafter. The objective of this intervention is to decrease the incidence of CD in the group receiving the gluten supplement compared to the group receiving placebo. It will probably be necessary to wait until the project's ending date, by December 2010 to know the effectiveness of these early dietary practices as a strategy to decrease the risk of CD development in children at risk.

The effect of environmental factors on future disease risk is relevant at the early stages of life when the immature neonate's gut undergoes the process of microbiota establishment and the immune system acquires full competence and tolerance to non-harmful antigens. The PROFICEL study, which we are currently carrying out, together with eight Spanish hospitals and several CD societies, is aimed at finding out environmental factors that might be involved in the development of CD in susceptible individuals. This study is being performed in a Spanish sample of infants at risk for CD (at least one first-degree relative with CD) and the global objective is to define the combined influence of the early environmental factors (dietary pattern and microbial exposure), the intestinal colonization process, the genetic background and the immune status of newborns and infants on the risk of developing CD (http://www.proficel.es). To this end, 200 recruited infants will be prospectively studied during at least 3 years registering early nutritional practices and clinic history, analysing the HLA status and other genetic markers, the immunocompetence and the intestinal microbial colonization pattern. Preliminary analyses have shown that an interplay exists between the HLA genes and the microbial colonization process[42] with some bacterial groups, such as total Gram-negative bacteria and *Bacteroides–Prevotella*, showing higher proportions in those infants with HLA-DQ genotypes associated with a higher risk of developing CD compared to those with intermediate or low risk HLA-DQ genotypes. Moreover, preliminary results on 100 infants have shown an interaction between milk-feeding practices (exclusive breast-feeding *v*. formula or partial breast-feeding) and HLA-DQ genotype on the proportion and absolute counts of lymphocyte subsets. We have observed significant interactions between HLA-DQ genotype and milk-feeding practices on some of the T CD8+ lymphocyte subsets analysed, such as memory and naïve CD8+ T-cells, CD8+CD38+, CD8+CD28+ and CD8+CD25+ (E. Nova, T. Pozo, Y. Sanz & A. Marcos, unpublished results). These findings might be relevant for the future immunological response to dietary gluten and merit further exploration after sufficient follow-up of the cohort has provided information on those children developing CD at some point. It will also provide extremely useful information once the study of the interaction between the microbial colonization pattern of the intestine in infants at risk and the immunocompetence development are analysed together as well as their possible influence on the final outcome regarding protection or promotion of disease development. However, the influence of the genetic background should always be considered. HLA susceptibility alleles for autoimmune diseases have been suggested to interfere with the thymic development of regulatory T cells including both CD4+CD25+ and CD8+CD25T reg (Foxp3+)[43,44]. On the other hand, a common genetic background (HLA and non-HLA genes) between CD and other autoimmune conditions[45,46] and the association of CD with other autoimmune disorders[29] support the influence of gene–environment interactions in the modulation of immunity and autoimmune disease risk.

Possible use of probiotics and prebiotics in infants at risk for celiac disease

It is generally accepted that the indigenous intestinal microbiota are able to modulate immune responses through the interaction with immune cells in the intestinal mucosa and to influence immune development in newborns and infants, while disturbances in the composition of the gut microbiota are believed to influence the pathogenesis of allergic disorders[47]. On this basis, considering that alterations in the microbiota of CD children have been documented[48], the administration of probiotics and prebiotics seems to be a good alternative to influence immune reactivity to gluten in CD subjects. However, if they really have a role, then it will more easily be exerted during the first 2 years of infancy when the immune network is being developed. It is worth remembering how intestinal bacteria, whether resident or transient, beneficial or pathogenic or signalling components derived from them, reach APC and interact with TLR expressed in different cell types in the intestinal mucosa with the possibility to direct immune responses and influence homeostasis. Moreover, recent studies demonstrate that probiotics improve the epithelial barrier function in various clinical settings. Preservation of tight junction protein expression, inhibition of epithelial apoptosis, decrease in pathogenic bacterial adhesion, reduction of pro-inflammatory cytokines and increase in mucus production and defensin secretion are some of the mechanisms that are responsible for the intestinal barrier-preserving effect of probiotic bacteria[49]. However, at present, no clinical studies in human subjects have been performed to assess probiotics or prebiotics in CD treatment or prevention.

Other emerging therapies involving gluten peptide modifications and toxicity neutralization

There are other dietary strategies being developed to block the immune response to gluten based on the identification of immunogenic epitopes and their suppression via enzymatic treatment or by using peptide analogues[50–52]. Gianfrani *et al*.[51] showed that the transamidation of wheat flour with microbial transglutaminase can be used to block the T-cell-mediated gliadin activity. Previously, several

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studies have shown that single amino acid substitution in the sequence of gliadin T-cell stimulatory peptides can decrease their binding affinity to the HLA-DQ2 molecule and abolish the immunogenicity of the modified peptide[53–55]. However, the great heterogeneity of toxic gliadin epitopes is an obstacle against the efficacy of this immunomodulatory therapy of CD[55]. On the other hand, Kapoerchan *et al.*[56] have shown that gluten peptides can be modified at specific positions, for instance introducing azide functionalities, without affecting their affinity for HLA-DQ2, and that these constructs can compete with native gluten peptides and prohibit recognition by HLA-DQ2-specific T-cells. These antagonist peptides might be a therapeutic option to treat CD patients as well as prevent CD development in infants at risk. However, this strategy would require that these competitive compounds were administered always accompanying any gluten ingestion, delivered intact in the small intestine and moreover, they should have a much higher affinity for the HLA-DQ2 molecule than that of the gliadin-derived peptides. There could be a different application for these modified peptides by using them in bread-making dough in substitution for wheat flour. However, the safest approach seems to be the enzymatic treatment of gluten with prolyl endopeptidases from different micro-organisms to detoxify gluten before reaching the small bowel[57]. These enzymes promote complete digestion of gluten protein preventing antigenic stimulation of the immune system. However, their stability under gastric conditions and their efficiency *in vivo* is still imprecise[58]. In fact, all of these are only emerging therapies in a developmental stage and no reports of human trials with these therapies have been published so far.

Conclusion

Dietary strategies to modify environmental determinants increasing the risk of CD development might be possible in future. Investigation of these possibilities will require time and human intervention studies, which could lead to dietary guidelines that are easily and safely conducted to prevent the disease.

Acknowledgements

This work was supported by grants AGL2007-66126-C03-01/ALI, Consolider Fun-*C*-Food CSD2007-00063 and AGL2007-66126-C03-03/ALI from the Spanish Ministry of Science and Innovation. The authors declare no conflict of interest. E. N. did the main writing; T. P., Y. S. and A. M. provided relevant advice and contribution to the manuscript content.

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https://doi.org/10.1017/S0029665110001825 Published online by Cambridge University Press