Send Orders for Reprints to reprints@benthamscience.ae

Current Protein and Peptide Science, 2018, *19*, 1-13

REVIEW ARTICLE

Celiac Disease: Historical Standpoint, New Perspectives of Treatments and Contemporary Research Techniques

Luciano A. Benedini[1,2,*], Noelia L. González Vidal[2] and María Alejandra Gonzalez[3]

[1]*Instituto de química del Sur (INQUISUR), CONICET - Universidad Nacional del Sur, Bahía Blanca, Argentina;* [2]*Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur (UNS), Bahía Blanca, Argentina. CONICET;* [3]*Planta Piloto de Ingeniería Química (PLAPIQUI), CONICET - Universidad Nacional del Sur, Bahía Blanca, Argentina*

ARTICLE HISTORY

Received: March 20, 2018
Revised: May 04, 2018
Accepted: May 26, 2018

DOI:
10.2174/1389203719666180531123514

Abstract: Celiac disease (CD) is an inflammatory syndrome that affects mainly the intestine, but also other organs. This ailment is also affected by the physicochemical behavior of gluten as such. From the medical standpoint, this pathology results from a combination of genetic and environmental factors. At the same time, gliadin (the alcohol-soluble fraction of gluten) along with other related oligomers, such as 33-gliadin, present high immunogenicity and are responsible for triggering of this disease. Within CD characterization, there are mainly two different approaches to carry out this study; one focuses on its chronic phase, while the other deals with its initial stages. Although the chronic phase of CD has been well characterized, the initiation of the inflammatory process is still unclear. As this process is apparently related to the aggregation of the oligomers involved in CD, the initiation of the disease could be explained by means of clarifying their self-assembly behavior. Thus, this work addresses the clinical explanation, within the chronic approach, attempting to combine it with the physicochemical techniques used for characterization of proteins aggregates as well.

Keywords: Celiac disease, environmental factors, genetic factors, immunologic disease, assembly of 33- gliadin, gliadin, spectroscopic techniques.

1. INTRODUCTION

Traditionally, the development of CD has been considered as a consequence of the essential interaction between gluten and both environmental and genetic factors [1]. However, a synthetic review of two relevant aspects of CD study is presented in this work. One of them is based on the clinical phase of this pathology, while the second one relies on the proteins aggregation assessment, which is responsible for the interaction with the intestine wall and CD triggering. The clinical aspect of the disease is described, in which its history, diagnosis, stages and treatment, along with new perspectives for this last issue, have been addressed. The history of the emergence of this disease was thoroughly studied in the literature, and it was initially described as a set of malabsorption symptoms [2]. Then, a relation between gluten and cell-mediated immune response in the small intestine was established [3]. Finally, the genetic predisposition was added to the environmental factor, as another important aspect involved in the development of CD, because it has been shown that patients with other pathologies (*e.g.* dermatitis herpetiformis) have the same human leukocyte antigens than those reported for CD [4, 5]. In this context, extra-intestinal problems can be associated with CD, and among them, neurological complications such as ataxia and epilepsy have been described [6-9].

The appearance of intra and extra-intestinal symptoms makes diagnosis an important phase of this disease. Consequently, a wide description of these different symptoms is carried out in this text. The evolution of patients, the type and proportion of world population affected by CD, and the role of immunologic system are also described. One of the most important immunological modulator peptides responsible for CD is the peptide 33-gliadin. In this context, the comprehension of complexity and chemical variety of proteins forming gluten is an essential starting point to reveal the forces which drive their behavior in the medium imposed by the intestine. Therefore, in this review, we evaluated the combination of the immunological system response with environmental factors after the interaction of gluten proteins with the gut's wall, in order to improve the understanding of the role of different actors in CD. Drago and coworkers reported that permeability of intestine wall is increased by presence of gliadin, irrespective of genetic predisposition of patients [10]. This behavior was also reported by Lammers and coworkers [11]. Later, Banc and coworkers have described interactions between the immunogenic actor, gliadin, and the intestine membrane [12]. However, these authors

*Address correspondence to this author at the Instituto de química del Sur (INQUISUR), CONICET - Universidad Nacional del Sur, Bahía Blanca, Argentina; E-mail: lbenedini@uns.edu.ar

1389-2037/18 $58.00+.00

analyzed only the interaction between proteins and the gut. Previous to this interaction, it would be necessary to assess proteins' behavior in the medium as such because gliadin, and its related peptides, can self-organize and develop different structures, from micelle-like to micro-fibrillar aggregates. Such organization of peptides depends on a combination of factors like their own concentration, medium ionic strength, temperature and pH values [13, 14]. It has been hypothesized that these aggregates of proteins may be implied in the development of the initial stages of CD and gluten sensitivity disease, when interacting with the intestine wall [13, 15]. Because this proteins' physicochemical behavior is so crucial in initial stages, it demands scientific attention and therefore, is a point of view specially reinforced in this text. The characterization of supramolecular structures formed by gliadin and related peptides could lead to one of the first steps towards the understanding of intolerance gluten disorders. For this reason, aggregation of proteins and the techniques for characterizing these aggregates and their folding (or miss-folding) are addressed, specifically those related with gliadin and 33-gliadin peptide.

2. CLINICAL ASPECTS OF CELIAC DISEASE: HISTORICAL PERSPECTIVE, CLINICAL CONSIDERATIONS, STAGES AND TREATMENT

2.1. Historical Perspective and Disease Prevalence

In a clinical scenario, CD can be described as a chronic enteropathy caused by the intolerance to dietary gluten, in genetically predisposed individuals. It is an immune-mediated disorder that not only affects the small intestine, but can also be considered a multisystemic disease [1, 16-21].

During the first and second centuries, Aretaeus Capadocia made the first reports about CD, describing an abdominal disease apparently related to nutrition. In 1887, Samuel Jones Gee provided the first CD classical features (symptoms and characteristics), noted that the disorder might occur at any age, and suggested that patients could be cured through an appropriate diet. It was not until 1953 that the paediatrician Willem-Karel Dicke recognized the role of wheat protein in CD. However, John W. Paulley conducted the first accurate description of CD intestinal lesions in 1954, through the examination of biopsy specimens. In the following years (1980s - 1990s) it was described the existence of antibodies and antigenic markers present in CD [4, 17, 20, 22-24].

CD prevalence varies across different countries, but it is estimated to affect approximately 1% of the population among Americans and Europeans, as well as in Australia, Africa, Middle East, India and probably northern China [4, 17-21, 24-27]. However, some higher prevalence values were detected, *e.g*. in Finland and Mexico prevalence range between 2% and 5%, and Saharawi (an African community) people presented the highest value (nearly 6%) of worldwide population [17, 19, 24]. On the other hand, CD disease is quite rare in East Asia and Pacific Islanders, because this population do not present genetic predisposition and/or have a low consumption of gluten [19]. CD occurs more often in women than in men, and in paediatric than in adults [20, 23]. However, Green and coworkers postulated that the greater prevalence in women is only valid when the clinical manifestations are taken into account. When serological screening is considered, the prevalence is comparable at about 1% [28]. Green and co-workers also based their conclusions on several facts including that autoimmune diseases are more common in women, more regular health care interaction performed in female than male subjects, and a higher likelihood of symptomatic disease among women than men [28]. Regarding the development of CD among relatives, the risk is much greater in first-degree relatives (up to 10%) and lesser in second-degree relatives [16]. In addition, the prevalence increased among individuals with different autoimmune pathologies, such as type-1 diabetes (3 to 16%), Hashimoto's thyroiditis (5%), psoriasis, vitiligo, inflammatory bowel disease and others [16, 17, 29]. As an example, the association of CD and autoimmune hepatitis was particularly studied, and a higher prevalence of CD in these patients was observed [29]. This was attributed to a same immunological basis in both diseases, so an advice for early serological screening testing for CD in these patients is of important concern [29].

In the last decades, there has been a significant increase in the number of new cases of CD, and 25% of newly diagnosed CD patients occurs in population older than 60 years [20]. This can be attributed to several factors, including increased clinician awareness, certain environmental factors, worldwide increase in wheat consumption, better diagnostic tests that can detect even subclinical disease and thorough screening of individuals considered to be at high risk [19, 20, 30]. Nevertheless, cases may continue to rise because CD still represents a statistical iceberg, where undiagnosed cases, represented below the waterline, are greater than the diagnosed cases, represented by the top of the iceberg [17, 30].

2.2. Peptides, Antigens and Main Actors for CD Development and Diagnosis

One particular peptide, the 33-gliadin, which corresponds to the 56-88 cleavage of α-gliadin, is especially rich in glutamine and proline, and it was found to be toxic and immunodominant [31]. This peptide is also highly resistant to gastric, pancreatic or intestinal (brush border membrane peptidase) degradation, due to its high proline content and lack of prolylendopeptidasic activity in human gastrointestinal enzymes [4, 31-36]. Additionally, it was found that no homologous sequences to the 33-gliadin peptide are detected in non-toxic proteins (rice, oat and maize) [37]. In the case of oat, it has been observed that CD patients tolerate it better, even though avenin can provoke a CD4-T cell response[4]. This can be explained by the reduced antigenic sequences present in avenin (in contrast with gliadin, secalin or hordein) and the much lower gluten content present in oat, compared to other cereals [4]. Besides, considering the primary structure of the protein (see section 3.1), T-cell responses against α- and ω- gliadins are evidently immunodominant in comparison with responses against γ-gliadins, which are less frequently observed [35].

Gliadin peptides, especially the described 33-gliadin, cross the intestinal epithelial barrier by paracellular tight junctions after the release of zonulin, a known physiologic modulator of intercellular tight junctions. Once inside the

cells, the enzyme tissue transglutaminase (tTG) deaminates gliadin, converting non-charged glutamine into negatively charged glutamic acid [4, 20, 31, 35, 38]. It is important to note that the tTG is mostly inactive in the intracellular environment, and a trigger factor is needed to release and activate it. This factor involves a pro-inflammatory scenario that could be determined by enteroviral infections or an initial response of T-cells to native gluten peptides [4, 35]. The generated deaminated peptides are more antigenic than native gluten peptides, and have greater affinity for human leukocyte antigen molecules (HLA-DQ2 or DQ8) on the surface of antigen-presenting cells [4, 19, 20, 23, 24, 31-33, 35, 36]. It is well established that those molecules (HLA-DQ2 and DQ8) prefer negatively charged anchor residues, in order to obtain an optimized interaction [35]. Peptides that bind to HLA-DQ2 show deaminations preferentially in positions P4 and P6 of α- and ω-gliadin, whereas binding to HLA-DQ8 are more related to negatively charged residues in positions P1 and/or P9 [35, 36]. This interaction leads to the activation of CD4-T lymphocytes in the intestinal mucosa and, in consequence, the immune cascade characterized by pro-inflammatory cytokines secretion and clonal B-cells expansion [4, 19, 23, 31, 35]. These responses cause an increase in natural killer cells, intraeptithelial lymphocytes (IEL) and cytokine levels (interleukines, interferon γ), which finally lead to intestinal damage, and secretion of anti-gliadin and anti-transglutaminase antibodies [17, 20, 21, 23, 31, 33, 35]. Besides, activated IELs lyse the epithelium and enhance the release of tTG, with subsequent gluten deamination and self-amplifying feedback loop involving T- and B-cells, which maintain inflammation and CD evolution [4, 35]. The characterization of these responses leads to the disease diagnostic ability, which includes serological tests and histological evaluation. Antibodies anti-tTG, anti-EMA (endomysial), anti-gliadin and anti-deaminated peptides can be used for these purposes. The IgA anti-EMA was used for clinical diagnosis of the disease because it has been observed that CD patients have autoantibodies to reticular fibres of connective tissue [35]. However, it was later identified that tTG was the antigen recognized by anti-EMA antibodies, leading to the diagnostic test of anti-tTG antibodies titre [35]. To date, the best strategy includes plasma quantification of IgA anti-tTG, with a sensitivity up to 97-99%, specificity approaching 96% and accuracy of 98%, following by further confirmation step that includes determination of IgA anti-EMA because of its higher specificity (around 99%), despite its reduced sensitivity (90%) [17, 19, 21, 23, 33]. In case of IgA deficiencies, IgA can be replaced by IgG evaluation, found in approximately 2-10% of CD patients [17, 23]. The anti-gliadin antibodies (IgA and IgG) are not actually recommended, except for children diagnosis, because of their low specificity, sensitivity and accuracy. They are replaced by the anti-deaminated peptide antibodies (IgA and IgG), which exhibit-sensitivities between 80 and 98% and specificities ranging from 86 to 96% [17, 19, 20, 23, 24]. Nevertheless, the gold standard for CD diagnosis is the duodenal biopsy, used to detect the histopathological changes (villous atrophy, crypt hyperplasia, decreased enterocyte height, increased IELs) and determine the Marsh classification of the CD lesions [17, 19-21, 23, 24]. In paediatric population, the biopsy can be avoided if certain parameters, including symptoms and levels of IgA anti-tTG and HLA-DQ2 in diagnostic tests, are met [19]. When diagnosis is inconclusive, a genetic analysis (HLA testing) can be performed, where it was observed that only a 0.4% of celiac patients are both DQ2 and DQ8 negative, and suggest (if positive) or reject (if negative) the potential CD diagnosis [17, 19, 21]. These markers have a high negative predictive value, so their absence effectively eliminates the possibility of CD [19, 20].

CD is triggered by both genetic and environmental factors (see Fig. **1**). It is well established that genetic factors play an important role in the development of the disease. In monozygotic twins the concordance for CD is about 80%, whereas in dizygotic twins the concordance is less than 20 %, indicating an important genetic link [39-41]. CD develops in genetically predisposed individuals, and is strongly associated with HLA class II genes known as HLA-DQ2 and HLA-DQ8, located on chromosome 6 [17, 19, 20, 24, 32, 42]. Approximately 90% of CD patients express the alleles DQA1*05 and DQB1*02, forming the HLA-DQ2 heterodymer [17, 19, 32, 35, 42]. Specifically, CD patients carry the DQA1*0501 α-chain and DQB1*0201 β-chain alleles in *cis* configuration on the DR3 haplotype, which encode the DQ2.5 molecule [43, 44]. The expression of DQ2.5 genes is an important risk factor in CD, because they have high affinity for the peptides formed from incomplete digestion of gluten and present gluten antigens to CD4+ T-cells, resulting in the intestinal inflammation, as it was previously stated [4, 39]. The remaining 10% of CD patients express HLA-DQ8 molecule, encoded by DQA1*03 and DQB1*03:02 alleles. However, the HLA-DQ2/DQ8 genes are common and are present in approximately 35-40% of individuals, but only 2-5% suffers from CD [1, 4, 17, 19, 32]. This indicates that the HLA-DQ genotype is necessary but not sufficient for the development of CD, and that other non-HLA genes might be involved and contribute to the disease. Considering this aspect, at least 39 non-HLA regions, that confer a predisposition to CD, have been identified [1, 4, 17, 19, 32, 43, 45]. On the other hand, it is extremely rare that HLA-DQ2/DQ8 negative individuals develop the disease [4]. To complete this scenario, it is important to emphasize that the intestinal immune system is the largest component of the immune system in the body [35].

As it was already introduced, the main environmental factor to develop CD is the exposure to gluten. There has been some hypotheses suggesting that breastfeeding and the timing of gluten introduction may influence the development of CD. Several studies have been conducted to corroborate these hypotheses, finding that there is no significant impact of these early nutrition practices on the risk of CD [32, 46, 47]. It was also stated that gluten might be introduced into the infant diet anytime between 4-12 months of age. In children at high risk for CD, earlier introduction of gluten (4 vs. 6 months or 6 *vs*. 12 months) is associated with earlier development of CD, but the cumulative incidence of each in later childhood is similar. On the other hand, the observational data point to the association between the amount of gluten intake and the risk of CD, consumption of large quantities of gluten should be avoided during the first weeks after gluten introduction [48]. Other risk factors include gastrointestinal infections, such as rotavirus in children and campylobacter in adults [49, 50]. Certain drugs, such as pump inhibitors and antibiotics have been associated with increased

risk of CD, although it is controversial because some of these drugs may have been given in response to symptoms caused by undiagnosed disease, rather than triggering its development [51].

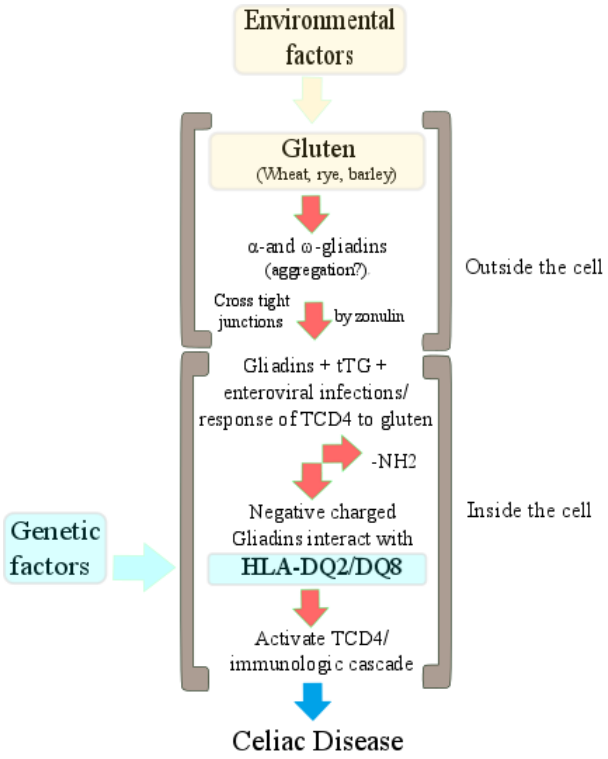

Fig. (1). contribution of environmental and genetic factors for the development of CD.

2.3. Clinical Manifestations

The clinical presentation of the disease is highly variable, and both the disease and its symptoms can appear at any stage of life [52]. CD can affect individuals from any age, including the elderly. In fact, more than 70% of new patients are diagnosed above the age of 20 years [52]. The Oslo definitions classify CD in several subtypes including classical (or typical), non-classical (or atypical), asymptomatic and potential CD [17, 53]. Signs and symptoms of malabsorption, such as diarrhea, steatorrhoea, and weight loss or growth failure, characterize the classical form. In non-classic CD, patients may develop non-specific gastrointestinal symptoms without signs of malabsorption, or with extra-intestinal manifestations (without gastrointestinal symptoms) [53]. Asymptomatic CD, or subclinical, include at-risk patients without symptoms, but with positive serologic results and villous atrophy on intestinal biopsies [20]. These at-risk groups include first-degree relatives with CD, and patients affected by Down syndrome or other autoimmune diseases [20]. Finally, potential CD relates to people with a normal small intestinal mucosa who are at increased risk of developing CD, as indicated by genetic predisposition and the presence of weakly positive CD serology [17, 53].

Clinical manifestation of CD can vary greatly, and signs and symptoms are different in children and adults [4, 20, 54]. In paediatric patients, manifestations can be either classical or non-classical, with vomiting, chronic diarrhea, swollen belly, poor appetite and recurrent abdominal pain [19, 54]. Older children and adolescents often present with extra-intestinal manifestations, such as delayed puberty, short stature, anemia, neurologic symptoms including attention-deficit/hyperactivity disorders, learning disabilities, lack of muscle coordination and seizures [1, 19].

Among adults, the classic presentation of the disease consists of a very marked malabsorption syndrome with postprandial abdominal pain and bloating, chronic diarrhea, and steatorrhea [19, 55]. However, more than half of adults with CD have signs and symptoms that are not related to the digestive system, including anemia, loss of bone density (osteoporosis), fatigue, reduced spleen function and other nutritional deficiencies such as vitamin D, folate, zinc, vitamin B12 and B6 [19, 52, 56]. Different types of neurological problems have been described among adults, such as peripheral neuropathy, ataxia and impaired cognitive function [19, 52]. Therefore, extra and intra-intestinal symptoms of CD are summarized in Fig. (**2**).

In genetically susceptible individuals, Hadjivassiliou and co-workers [7] have reported that gluten ataxia is an immune-mediated disease triggered by gluten ingestion which implies a cerebellar involvement. This ataxia is defined as sporadic idiopathic cerebellar ataxia related with anti-gliadin antibodies, IgA and/or IgG type, discarding other aetiology for ataxia. This pathology has a prevalence of 15% amongst all ataxias, and 40% of all idiopathic sporadic ataxias [8]. Other neurological problems have been reported by Briani and coworkers [57]. These issues include headache, depression, entrapment syndromes and epilepsy. Gerace and co-workers [9] reported that gluten free diet provided to patients with this last neurological problem generates a reduction of both seizure frequency and doses of antiepileptic medication. The authors reported a possible mechanism underlying the relationship between gluten-related disease and epilepsy, indicating that a potentiation of kainate-induced neurotoxicity through the peptide gliadin 31-43 links the toxic effects of gluten to epilepsy [9]. Lastly, an uncommon skin manifestation known as dermatitis herpetiformis is a unique presentation of CD, predominately detected in adults, and affects about 10% to 20% of patients [17, 20].

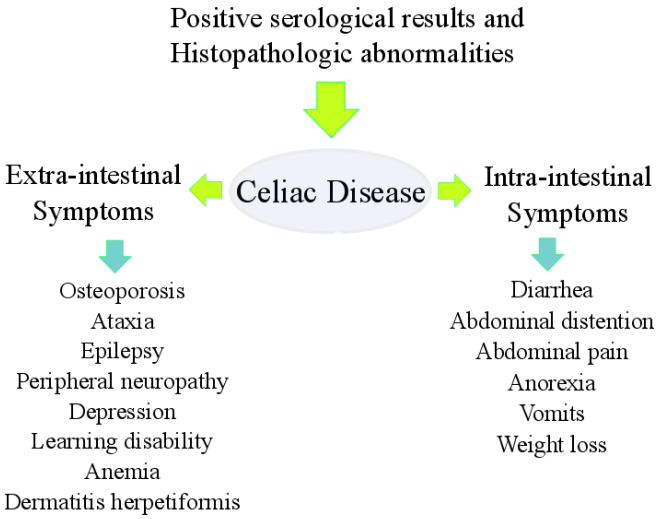

Fig. (2). Extra and intra-intestinal symptoms of CD.

2.4. Treatment

Although there is no pharmacological treatment for CD, it can be successfully managed following a strict lifelong gluten-free diet (GFD), which leads to the relief of symptoms, healing of intestine, reverse of serological results and consequences of malabsorption, and enables the patient to maintain a healthful and nutritionally diverse diet [20, 58]. Patients have to exclude from their diet all foods containing wheat, rye and barley, and their derivatives, and include foods that are naturally gluten-free (fruits, vegetables, dairy products, meat, fish, poultry, nuts, pulses, eggs) or manufactured gluten-free products (bread, pasta, flours, cereals, desserts) [18, 58]. The amount of gluten intake should be less than 10-50 mg/day, a value considered safe and unlikely to cause mucosal abnormalities [16, 58]. Currently, the International *Codex Alimentarius* defines gluten-free foods as the ones containing less than 20 ppm of gluten, which allows a safety margin for the variable gluten sensitivity of patients [16]. Another important regulation include the Food Allergen Labelling and Consumer Protection Act, where it is stated that all food that contain any of the top eight allergens (milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, soybeans and wheat) should be strictly labelled [20]. However, rye and barley are not included in these top eight allergens, and could be source of hidden gluten [20].

Under a strict GFD, clinical improvement is achieved within a few weeks, while the mucosal recovery takes a longer time (1-3 years) [17, 19, 20, 24]. It has been observed that recovery rates are higher in children than in adults, with greater rates of improvement in gastrointestinal over extraintestintal symptoms; and regarding sex, better rates of symptoms remission were detected for males compared to females [59]. Some investigations suggest that a GFD may also relieve symptoms in patients with other related diseases (*e.g.* systemic lupus erythematosus, dermatitis herpetiformis, irritable bowel syndrome, rheumatoid arthritis, type 1 diabetes, thyroiditis, psoriasis, and autism spectrum disorders), and could have beneficial effects over infertility and related complications of pregnancy due to CD [24, 27]. However, despite the benefits of a GFD, maintaining this therapy for a long term is a difficult issue, especially in teenagers and adults. This could be attributed to several difficulties of GFD (*e.g.* adherence, cost, nutritional imbalances, restrictions on the social situation), which result in a decreased quality of life [60].

It has to be pointed out that is really difficult to avoid gluten completely because it is ubiquitous, especially in food industry where it can be found in common products associated with wheat, as well as a hidden ingredient [4, 61]. Gluten can be detected even in medication, when starch is used as excipient [26]. In this scenario, several regulatory agencies have been established the information that should be added to labels and patient leaflets, for the awareness of gluten presence [26].

Secondly, gluten free foods are significantly more expensive (three times) [62-65] than gluten containing foods [66]. In this context, governments of different countries provide several policies to ensure equal access for all patients to a set of basic gluten free products. For instance, CD patients get tax deduction or monthly allowance or vouchers to buy gluten free foods [18].

Thirdly, gluten-free products are poor sources of vitamins, fibres and minerals compared to gluten-containing ones. Patients adhering to GFD may have low levels of fibres, folate, magnesium, calcium, zinc, iron, vitamin B12 and vitamin D [19, 67]. Lack of these nutrients can result in other medical complications, such as osteoporosis or anemia. Lastly, GFD was reported to modify the composition and immune properties of a gut microbiota in adults, with particular reduction in beneficial gut bacteria [27, 61, 64]. All these observations contradict the idea that GFD is beneficial for healthy individuals, because nutrient deficiencies and possible overweight are developed, and beneficial properties of gluten (reduction of triglycerides level, blood pressure control *via* inhibition of angiotensin I-converting enzyme, reduction of cancer risk) are lost [27].

As a final remark, concerning the current treatment options, it is important to consider that the adherence to GFD also depends strongly on patient motivation and education, where the advice of experienced dietitian, support of family members and self-help groups become essential to successful adaptation to celiac lifestyle [19, 20].

Currently, no medical treatment is approved for CD [19]. Glucocorticoids can improve symptoms of CD patients, but are not recommended because of their substantial side effects [19, 21]. Besides GFD and use of pre-treated flour for manufacture of gluten-free products, other approaches for CD treatment have arisen.

2.5. New Perspectives of Treatments

Other approaches for CD treatment include modified grains, blocking of gluten passage across the intestinal membrane, inhibition of intestinal TG with specific blockers, immunotherapy, vaccines and gluten-degrading enzymes [17, 24]. The first one, modified grains, implicates the mutation or silencing of immunostimulatory protein sequences, although ethical implications about genetically modified foods arise [17, 38]. In the second approach, an agent called larazotide (AT-1001) can be identified, that functions as a zonulin inhibitor (*i.e.* decrease tight junction permeability) [17, 21]. Considering the third option, it was postulated that a possible modulation or blockage of tTG activity would prevent the immune cascade characteristic of CD, although it could present undesirable side effects with a long-term use [23, 24, 33].

The most promising approach is the gluten-degrading enzymes because gluten has high proline content and human gastrointestinal enzymes lack prolylendopeptidasic activity, resulting in incomplete digestion of gluten and generation of characteristic immunogenic peptides [17, 32, 34]. In this case, the intestinal microbiota may play an important role, considering the recent literature data suggesting that bacteria could be involved in gluten hydrolysis [19, 32, 65, 68, 69]. In support of this hypothesis, the early use of antibiotics has been linked to a higher risk of developing CD [19, 69]. In healthy conditions, the microbiome plays an essential role in food metabolism and performance of immune systems, and helps to maintain the gut conditions with no development of

inflammatory reactions to food or bacterial antigens [69]. However, dysbiosis could promote loss of oral dietary tolerance, both by the reduction in short chain fatty acid production or by a *Proteobacteria* expansion, leading to reduced immune tolerance and intestinal inflammation respectively. Nevertheless, it is not totally elucidated whether the microbiome is causative for disease, the disease causative of dysbiosis or if it is a combination of both [69].

Several studies were conducted in order to characterize the intestinal microbiota of healthy and CD individuals, with contradictory conclusions, and may indicate that pathophysiological implications of intestinal bacteria in CD remain unknown. Most of these studies were carried out in samples of the oral cavity or feces, and bacterial strains with gliadin-peptides digesting capacity were isolated [32]. One particular study, conducted by Herran and coworkers, characterized the microbiota with gluten-degrading capacity in the specific site of development of CD, *i.e.* the small intestine (duodenal biopsies), which is a hostile habitat for bacteria due to low pH, rapid peristalsis and presence of bile salts, but an excellent nutrients source [32]. Herran and coworkers and other research groups found four phyla of bacteria, including *Firmicutes* (73-88%; mainly lactic acid bacteria like *Lactobacillus*, *Enterococcus*, *Streptococcus*), *Actinobacteria* (8-15%), *Proteobacteria* (3-12%), and Bacteroidetes (1%), and also species that showed extracellular glutenasic activity included strains of *Bacillus* sp., *Staphylococus* sp., and *Pseudomonas aeruginosa* [32, 68, 70]. It is important to point out that the found microbiota was not common between individuals, and isolated species were characteristic of each volunteer [32]. *Lactobacillus* was the main bacterial group isolated, independent of healthy or CD individuals, while opportunistic strains (*e.g. Pseudomonas aeruginosa*) were predominant in CD patients [32]. Besides, genera like *Actinomyces*, *Bacillus*, *Lactobacillus* and *Pseudomonas* showed hydrolyzation activity over gliadin proteins and the 33-gliadin peptide, with different degradation pattern between them (*i.e*. different proteases activity) [32, 68, 70]. However, not all bacteria implicated in gluten metabolism are health promoting. Bacterial proteases of some groups (*S. epidermis*, *E. faecalis*, *E. coli*, *C. perfringens*, *C. sordellii*) may be related to inflammatory bowel disease, and the specific proteolytic activity of *P. aeruginosa* over 33-gliadin peptide generates smaller peptides with preserved high immunogenicity [68, 70]. However, these immunogenic peptides produced by *P. aeruginosa* elastase can be further degraded by *Lactobacillus*, and reduce its potential intestinal toxicity [68]. Thus, further *in vivo* studies are needed in order to completely elucidate the role of these bacterial groups in gluten hydrolysis, immunogenicity and CD pathogenesis, before they can be proposed as therapeutic or pharmacological alternative for CD treatment [32, 68].

A supplement therapy with bacterial prolyl-endopeptidases has been proposed to improve gluten digestion in the gastrointestinal tract and destroy T-cell epitopes, although the real *in vivo* usefulness of this strategy is still under revision [17, 33, 65]. Results with several candidates (including a prolyl-endopeptidase derived from a flavobacterium) suggested the potential therapeutic efficacy of this approach to the treatment of CD, implying the degradation of specific peptide sequences and the consequent inability to generate immunogenic epitopes via tTG [38, 71, 72]. All these results suggests that oral enzymatic supplementation could be a promising strategy, as peptidases allow the degradation of toxic peptides and detoxification of gluten before they reach the intestinal mucosa [21, 34, 72, 73].

Special interest was focused on *Lactobacillus* bacteria, as they have developed complex proteolytic and peptidolytic activities, and were demonstrated as one of the bacterial groups with most efficient gluten degradation capacity, *i.e.* detoxifying gliadin [32, 68]. *Lactobacillus* can be a transitory resident in the small intestine and provided from fermented foods, which could lead to temporary gluten degradation capacity [32]. The term 'probiotic' implies the administration of suitable quantities of live microorganisms (that should be alive in food or supplement, survive the severe gastrointestinal conditions and adhere to intestinal epithelial cells) to provide a health benefit [74, 75]. Probiotics are widely used for other pathologies, such as prevention of colon cancer, lowering cholesterol and blood pressure, management of lactose intolerance, preventing harmful bacterial growth under different pathological situations, among others [73]. Thus, administration of *Lactobacillus*, especially those with 33-gliadin peptide hydrolytic activity, as a probiotic agent in CD patients may improve the response to GFD treatment and improve the gut microbiota dysbiosis and the chronic intestinal inflammation [32, 75]. Likewise, enzymes from other genera, with proteolytic activity against 33-gliadin peptide, as well as against the whole gluten molecule, could be purified and used to eliminate gluten traces in food and beverage, both before or after consumption [32]. Moreover, it was postulated that the administration of *Lactobacillus* as a probiotic may improve the reduction of beneficial bacteria observed in CD patients adhering to the GFD (associated with overgrowth of opportunistic pathogens, like *E. coli* and total *Enterobacteriaceae*), and restore the gut ecosystem in treated CD patients [70, 75, 76]. Some species of *Bifidobacterium* (*e.g. B. longum*) is also available as a probiotic food supplement, considering its ability to hydrolyse the immunogenic 33-gliadin peptide [68, 74]. Nevertheless, in the case of probiotics, further studies should be conducted in order to evaluate the effectiveness of the particular agent along the gastrointestinal tract and its safety related to dose [34]. And the most important remark is that probiotics are not intended to replace GFD treatment, but to supplement it, to prevent effects due to inadvert gluten consumption, and to attenuate the altered inflammatory parameters and microbiota dysbiosis [75].

Recently, new approaches related to medical treatments of CD are described in the scientific literature. An example of the alternative approach that targets the presentation of gluten epitopes to T-cells is the inhibition of cathepsin S, an endopeptidase (cysteine protease) that may degrade antigenic proteins to peptides for presentation to the MHC class II (HLA type). Clinical trials were performed with RG7625, a cathepsin S inhibitor, on the immune response to a gluten challenge in volunteers with CD [36]. In the case of immunotherapy, some antibodies against inflammatory mediators have been evaluated for treatment of other diseases, like inflammatory bowel syndrome or Crohn´s disease, but further studies are recommended for extension to CD [17]. Clinical trials have been started with vaccines based on gluten pep-

tides recognized by HLA-DQ2 [17, 24, 77]. CD represents an ideal model for the potential evaluation of epitope-based immunotherapy because the amino acids (AAs) sequences recognized by T cells are well characterized, the gluten epitope hierarchy is preserved in most patients and the reactive T-cells can be mobilized from peripheral blood after gluten exposure and quantified by IFN-γ release [78]. One important contribution in this field involves the development of Nexvax2, and adjuvant-free mix of three synthetic highly soluble peptides with epitopes targeting CD4-positive T cells, in order to turn them insensitive to further antigenic stimulation [36, 77]. However, this immunotherapy scheme could be applied to majority but not all celiac patients, because it is shown to be responsive only in those who have both a HLA-DQ2.5 haplotype and a positive whole blood IFN-γ release assay [78]. In summary, although promising results were obtained with vaccination, there are some drawbacks that still need to be resolved, *i.e.* efficacy, side effects (safety), affordability, protection against CD complications (*e.g.* refractive CD) and optimal route, dose and regime of administration [17, 21, 77, 78].

3. COMPOSITION AND CHEMISTRY OF GLUTEN: CHEMICAL PROPERTIES AND PHYSICOCHEMICAL. ASSESSMENT OF AGGREGATION OF GLIADIN AND RELATED PEPTIDES

3.1. Composition and Chemistry of Gluten: Gliadins and Glutenins

Gluten is a rubbery mass obtained after the removal of the starch granules and the aqueous soluble portions, from wheat and similar grains. It is formed by proteins (70-85%), lipids (5-10%) and carbohydrates [79]. Gluten proteins are one of the most studied protein-complex of nature due to their components, size, and the characteristics of the sources, such as genotype, growing conditions and the technological processes to which gluten is exposed. The components of gluten can be divided in two main portions: gliadins and glutenins. Gliadin and glutenin constitutes the prolamins of gluten, and it is well-known that gliadins are one of the main environmental factors implicated in CD [4, 31, 80]. It represents a 40% of wheat protein, associated with an infrequent AA composition, with prevalent content of glutamine (around 37%) and proline (around 17%), and low levels of tryptophane (0.4%) and AAs with charged side chains (*e.g.* lysine 0.8%) [4, 80]. Prolamins can be classified considering the solubility of each of these parts into alcohol aqueous solutions; where gliadins are soluble and glutenins are insoluble. These proteins contained in gluten might be available as monomers, dimers and forming oligomers or polymers linked by disulphide bonds [81].

Initially, the gliadins are mainly presented as oligomers, and they are classified depending on their mobility in electrophoretic gel and their primary structure. They can be divided in four categories, in which the α-gliadins have the highest mobility, and in decreasing order are: β-, γ- and ω-gliadins [80]. Recently, using newer characterization techniques, a hundred of components have been identified and the gliadins have been re-classified into: α/β-, γ-, ω5- and ω1,2-gliadins. The α/β-, and γ-1,2-gliadin classes are more abundant compared to the ω –gliadins and they have similar molecular weight, between 28000 and 35000 D [82]. However, they differ in some AAs, such as tyrosine, and the repetitive units of peptides. The α/β-gliadins have repeated units of dodecapeptides, and γ-glidins of heptapeptides. The numbers of cysteins in the *C*-terminal, which confer intrachain crosslinking, also differ between these gliadins [83].

The ω-gliadins have a molecular weight between 40000 D (ω1,2-) and 50000 D (ω5-), and high contents of glutamine, proline and phenylalanine characterize these gliadins. In general, disulphide bonds in ω-gliadins are absent, or present as intrachain bonds [79].

In all described gliadins*, N*-terminal domains adopt β-turn conformations,. whereas *C*-terminal domains adopt α-helix and β-sheet conformations [83].

It has been described in the literature that gliadins highly contribute to the gut toxicity in CD.. α- and γ-Gliadins possess four and five different domains. The domain I of α-gliadins is rich in glutamine, proline and aromatic rings, and Weiser has reported that this domain is responsible for the activation of CD [80]. The presence of α-gliadins does not change in different species of wheat and therefore, neither in its toxicity [80].

The α-gliadins represent 15-30% of the total protein content of wheat, with a total of 282-286 AAs and a globular structure [31] . However, not only gliadin is characterized by the described AAs composition. Secalin and hordein, prolamins of rye and barley, are also characterized by a high composition in glutamine (around 37%) and proline (17-23%) [31, 80]. Both, glutamine and proline are considered substantial for CD development [31, 80, 84]. The intact three-dimensional structure is not relevant for the disease, as gliadin is digested in the human gastrointestinal tract (by pepsin, trypsin, chymotrypsin, carboxypeptidases, elastases nor pancreatin), and the obtained peptide mixture retains the celiac toxicity [34, 80]. The AAs sequences identified to be essential for the toxicity are PSQQ (Pro-Ser-Gln-Gln) and QQQP (Gln-Gln-Gln-Pro) are found in toxic prolamins (wheat, rye and barley) but not in non-toxic cereals (rice, maize) or other food proteins (milk) [38, 80]. Gliadin can be enzymatically degraded into different portions. One of them is a peptide formed by 33 AAs, 33-gliadin, which remains intact despite of a prolonged proteases exposure. Additionally, three distinct patients-specific T cell epitopes were identified for this peptide [72], indicating that 33-gliadin is an immunologic modulator.

3.2. Aggregation of Gliadins and Related Peptides

Several aggregation studies of gluten, gliadins and other peptides, have been reported [85-87]. One of the aims of these studies was to establish a relation between aggregation processes and the interaction of those aggregates with the intestine wall.

Proteins are formed by sequences of AAs, which confers the primary structure. Depending on the AA composition, proteins acquire a secondary structure. This three dimensional structure is produced by interactions through hydrophobic bonds, hydrogen bonds and electrostatic forces among AAs. When proteins are dissolved in aqueous medium, they fold and their hydrophobic portion turns inside

(to their non-polar core) and the polar portion turns outside, to interact with the solvent. Proteins interact with a wide range of molecules such as other proteins, carbohydrates, inorganic molecules, lipids, among others, and these intermolecular interactions also contribute to the establishment of the secondary structure of proteins and their function. The knowledge of the secondary structuremay lead to the understanding of the behavior of proteins under physiological conditions.

Protein aggregation is a natural phenomenon, usually associated with a miss-folding process [88]. This event is driven by numerous biochemical factors and different mechanisms [89]. Many of these mechanisms are not exclusive for proteins, and its understanding could be the key for solving related diseases [89]. The three dimensional structure of proteins is defined after the translation and the aggregation. One of the main mechanisms of protein aggregation is the reversible association of proteins in their native form. Among the surfaces of different protein monomers, a certain complementarity exists, and consequently leading to formation of oligomers throughtheir interaction [90, 91]. However, the proteins may undergo conformational changes and miss-folding (or partial miss-folding), resulting in strongly bonded monomers. This modification of the native proteins can occur under stress conditions, such as a temperature increase, and is described as an aggregation of protein in non-native form [92]. Another aggregation process occurs when a native protein is chemically modified, which generate conformational changes and therefore, the monomers aggregate. Some differences can be detected between these mechanisms. The first mechanism is a reversible process, until a higher mass aggregates of oligomers is generated. This process may become irreversible if mass of aggregates is increased. The second and third mechanisms imply conformational changes forcing the protein into non-native form, resulting in irreversible changes. Another mechanism that describes protein aggregation is based on the generation of a nucleation focus [89]. Hence, nucleation-controlled aggregation can be explained in the same way as the crystals grow in a saturated solution. Native monomers have low tendency to form aggregates and are more prone to precipitate. I If a low mass of monomers nuclei are formed, the probability of raising their mass leading to precipitation increases. This mechanism is called homogeneous nucleation. If the nucleus is not formed by native protein monomers, the process is called heterogeneous nucleation. In this case, an impurity, contaminant or another substance can act as nucleation seed, and the growth of oligomers of the native protein and their precipitation are due to that fact. The third mechanism described in the literature is the aggregation mechanism mediated by surfaces, according to which, the native monomers interact with a surface and the aggregation begins. Hydrophobic and electrostatic forces, which modify the conformation of the protein, are involved in this mechanism. The surface-induced modification generates a partial unfolding and, consequently, aggregation [88, 89, 91, 93-95]. The understanding of mechanisms leading to protein aggregation could explain the processes that occur in the preparation of protein-containing foods and also clarify the effects that occur inside the gut which affect humans.

Aggregation behavior of α-gliadins has been reported by Kasarda and coworkers [85]. This study has shown that aggregates of gliadin are formed at pH 5, when the ionic strength is increased. This behavior is driven by non-polar residues of gliadin which induces hydrophobic interactions [85]. Cole and coworkers have reported that the formation of α-gliadin aggregates depends on pH, ionic strength and temperature [87]. At pH 3, α-gliadin elutes as a monomer after passing through a chromatographic gel column. At pH 4 or 5 α-gliadin aggregates with aggregate molecular weight of approximately 1.10^6 D. Additionally, an increase of ionic strength from 0.005 to 0.01M progressively generates aggregation. When pH values are kept between 4 and 5, and temperature is increased, the aggregates start to dissolve. However, under these same pH and temperature but with increasing the ionic strength, the aggregates become more stable. In addition to the hydrophobic forces reported by Kasarda *et al.*, Cole *et. al* reported that hydrogen bonds also contribute to the formation of aggregates, whereas electrostatic forces have less importance under conditions in which temperature and ionic strength are changed [85, 87]. The ionization of carboxyl groups is affected by pH, and induced electrostatic repulsions play a significant role in the formation of secondary or ternary structures of gliadin. Hydrophobic forces, ionic interactions and hydrogen bonds maintain the globular structure of proteins. This implies that secondary structure of this protein is turned into a very ordered form. Other research groups have supported the hypothesis that one of the first steps in the development of CD is a non-immunogenic step [96]. In addition to gliadins' including high resistance to proteolytic degradation, the hydrophobicity has been reported as the most important physicochemical property [96]. This property is related to the forces that play a key role in their aggregation and the formation of complexes between gliadins and other proteins, such as HLA-D gene products, at intestinal level. Consequently, this interaction in genetically predisposed individuals can lead to CD [97].

3.3. Methods for Assessing the Aggregation and Folding Process of Gliadins and Related Peptides

A proper selection of the technique for assessing the aggregation and secondary structure of proteins depends on the resolution level and simplicity of interpretation of generated data. Nuclear magnetic resonance (NMR) achieves a high resolution, but the interpretation is arduous and the samples must be highly concentrated. For this reason, less complex techniques, such as UV-Vis spectroscopy are desirable to study protein aggregation, although the resolution is lower than NMR [98].

UV-Vis is a simple, fast, cheap, non-destructive and sensitive technique for analyzing samples in aqueous solutions, and also in other solvents (which should not interfere with the absorption spectrum of the sample). It is a well suited technique for quantitative measurements of a small amount of samples, owing to its sensitivity. The spectrum is obtained when a portion of the electromagnetic energy of the incident beam is absorbed by the sample, and electrons are carried to a higher energy state (*i.e.*, electrons are promoted from a basal state to an excited one). The absorption wavelength is affected by chemical features (presence of chromophores), analyte concentration, and by the sample matrix. This tech-

nique has been applied to the evaluation of conformational transitions of proteins [99]. Although this spectroscopy has low resolution, it is still very useful for complex systems, such as proteins [100]. The absorbance of proteins is essentially based on the absorption of (AAs with aromatic rings, such as tyrosine (Tyr) and tryptophan (Trp), which absorbs at 275 and 280 nm, respectively. Due to the high content of Trp the absorbance of gliadin in UV region can be measured. In addition, the absorption of Tyr and Trp depends on the polarity of the medium allowing studying of protein conformational changes by UV-Vis spectroscopy. When aromatic AAs-containing proteins fold naturally and these non-polar aromatic AAs moieties arrange towards the hydrophobic core of the protein, a bathochromic shift is generated. This change is clearly observed when the protein is unfolded, and the absorption wavelength returns to values characteristic for aromatic AAs. One important characteristic of this technique is the possibility to detect short-living structures, which correspond to intermediate conformations adopted by proteins during the interactions with other components in solution. Torrent and coworkers have reported the use of the fourth derivative UV-Vis spectroscopy for determining the folding of a prion protein, under different pressure and temperature [99]. When a protein is unfolded, hypsochromic shift of the derivative band occurs between 270 and 280 nm. Herrera and coworkers studied the self-assembly of 33-gliadin peptide by UV-Vis spectroscopy [13]. In the reported study, the absorption wavelength of Tyr is shifted from 274.5 to 275 nm when the concentration of this peptide is increased. This behavior is assigned to a reduced interaction between Tyr and the solvent due to the self-assembly of the 33-gliadin.The authors demonstrated by UV-Vis spectroscopy in combination with other techniques that gliadin aggregates when pH of the medium is changed from 3 to 7, thus preventing 33-gliadin's enzymatic degradation.

Additional techniques such as circular dichroism (CDic) may also be used to gain a better understanding of proteins aggregation and folding.. CDic is used for rapid determination of the secondary structure of peptides, proteins and DNA. CDic measure the difference between the absorption of light polarized circularly to the left (A_L) and that polarized to the right (A_R). The absorption bands of peptides and proteins, produced by a modification in their conformational distribution, are located in near and far-UV. For example, the absorption spectrum of amide group ranges from 170 to 250 nm, and for aromatic rings from 250 to 300 nm [101]. The CDic spectra of secondary structure of proteins, such as α-helix, β-sheet and polyproline II-like helix, range from 178 to 250 nm. Conformational changes of molecules are produced when interactions with other substances occurs. Additional alterations are also produced by the naturally trigged folding or by denaturalization of proteins. Conversely, the concentration of each sample constituent affects directly the signal of CDic. Thus, the resulting spectrum is the sum of these issues.

As previously mentioned, α-gliadin aggregates when the ionic strength (of a pH 5 solution) is increased. CDic studies can be carried out in order to evaluate whether a conformational change is produced during the aggregation process. Kasarda and coworkers reported that no corresponding changes associated with the peptide bonds were found, and thus no major conformational change occurs when this protein aggregates [102]. This technique was also used for studying the secondary structure of the peptide 33-gliadin which adopts a polyproline II conformation as this conformation is associated with aggregation process [72. In another work that reported the use of this technique to to study the aggregation of gliadin at the physiological pH of the digestive tract, showed that the absorption wavelength of Try shifts from 280 to 282 nm, depending on the experimental conditions [14]. Herrera and coworkers have reported an equilibrium between polyproline II conformation of 33-gliadin peptide and β folded structure [13]. The authors demonstrated that concentration, ionic strength and pH of solution play an important role in self-assembly processes. In this work, CDic was combined with molecular dynamics calculations and electron microscopy, to show that an initial aggregation of two molecules of 33-gliadin (which forms a dimer) is the first steps in this peptide self-assembly. In a medium with a higher ionic strength, 33-gliadin aggregates into nanospheres, but the decrease of the ionic strength of medium, leads to fibril formation. This behavior has been also reported in the literature for gliadin solutions [85, 87]. The forces that drive this effect could be a combination of hydrophobic effects, hydrogen bonds and electrostatic charges, due to the high content of proline and glutamine. In addition, this effect can be interpreted as an "early stage" of interaction between the aggregates and gut mucosa and a possible step toward the development of inflammation.

Another technique that can be used for studying aggregation and folding of proteins is the fluorescence spectroscopy. This technique is highly sensitive for assessing changes of proteins folding and conformational dynamic stages [103]. Fluorescence spectroscopy is based on measures of the amount of photons released by molecules with fluorescence capability. Molecules studied by this technique are named fluorophores. A fluorophore can be the sample itself, or fluorophore moiety can be introduced into the sample. Trp, Tyr and phenylalanine (Phe) are AAs responsible for proteins autofluorescence. Trp fluorescence is the most frequently used for studying proteins folding, because Tyr and Phe have low extinction coefficients and low quantum yields resulting in the relative low environmental sensitivity of their emission energies [104]. Nevertheless, fluorescence of Tyr and Phe can also be useful in conformational studies because large changes in proteins conformation modify the fluorescence intensity of these AAs. Calderon and coworkers used fluorescence spectroscopy to study the formation of associations between gliadin and other proteins in the intestinal tract [96]. They have shown that the formation of these aggregates play an important role in the development of CD in sensitive individuals. Others have also assessed the aggregation of gliadin under physiological conditions by the evaluation of the emission spectrum of Trp [14]. These reports showed that colloidal particles are formed at pH 3, similar to micelles structures, whereas at pH 7 condensed nanoparticles are obtained. The obtained nanoparticles showed high resistance to enzyme degradation. Dynamic Light Scattering (DLS) is one of the most powerful techniques used to study particulate systems because it can show a profile of particle size distribution in solution [105-107]. Thus, it is used to describe the aggregation behavior of proteins, polymers, surfactants and

carbohydrates. DLS is based on the temporal fluctuations of light scattered by the particles at given scattering angle [106, 107]. This parameter contains information about the movement of the particles, more precisely, the translational diffusion coefficient (d_t). The information of the dynamic system is expressed by the function g_1, which is a normalized electrical field correlation.. Structural transformations are related to the counts per second hitting the detector after passing through the sample, and changes in the hydrodynamic radii of the aggregates can be assigned to the increase of assembled units. DLS have been used for studying the associative behavior of the 33-gliadin peptide [13]. The authors showed that the behavior of this system can be related to surfactants systems yielding micelles. This means that under This report shows that at certain concentration of 33-gliadin no association occurs, but when the critical molecular concentration is reached, the peptide associates by forming different type of aggregates. When the concentration of 33-gliadin peptide range is between 125 and 610 μM but the ionic strength of these solutions is changed, the system shows oligomers coexisting with larger aggregates such as fibrils. Similar properties were reported for α-gliadin [13].

Techniques for assessing aggregation of 33-gliadin and factors which modify this behavior are summarized in Fig. (**3**).

In summary, α-gliadin and 33-gliadin peptide aggregate at physiological conditions and formation of these aggregates could explain the non-immunological phase of initial stages of CD in sensitive patients. However more studies are required to confirm this hypothesis.

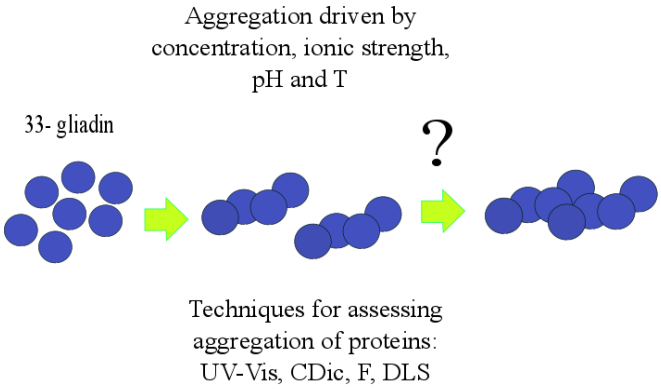

Fig. (3). Scheme of aggregation of 33-gliadin, techniques and considerations for studying this behavior.

CONCLUSION

This review describes two important aspects of CD. The first aspect is related to clinical considerations, from diagnosis to traditional treatment of the disease, also addressing updated views and perspectives of new treatments. The second aspect is based on the physicochemical properties of proteins involved in CD pathology, and the interaction among them and with other molecules. The studies described herein provide a basis for understanding the key mechanisms that govern the development of CD. The aggregation of gliadins and related peptides is considered as a part of environmental factors, and as such, effect of these proteins and peptides aggregation should be evaluated before the immunological factors. Studies of aggregation of these proteins could be used to explain the initial stages of the disease. Thus, with this aim in mind, this review describes techniques commonly used to study proteins folding and aggregation that can be applied to analysis of structural properties of gliadin, including 33-gliadin peptide, before its interaction with the intestine wall. The continuous advances in both fields, clinical and physicochemical, are crucial to improve diagnosis, evaluate new treatments, and expand knowledge on aggregation process of gliadins in the intestine, that will lead to improvement in CD patients' life quality.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors acknowledge the support from Universidad Nacional del Sur (PGI 24/Q064 and PGI 24/ZB70). Luciano Benedini and Noelia Gonzalez Vidal are researchers of CONICET. Maria Alejandra Gonzalez has a Fellowship of CONICET.

REFERENCES

[1] Green, P.H.R.; Cellier, C. Celiac disease. *N. Engl. J. Med.,* **2007**, *357*(17), 1731-1743.

[2] Dicke, W.K.; Weijers, H.A.; Van De Kamer, J.H. Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta Paediatr.,* **1953**, *42*(1), 34-42.

[3] Ferguson, A.; MacDonald, T.T.; McClure, J.P.; Holden, R.J. Cell-mediated immunity to gliadin within the small-intestinal mucosa in coeliac disease. *Lancet,* **1975**, *1*(7912), 895-897.

[4] Tjon, J.M.-L.; van Bergen, J.; Koning, F. Celiac disease: How complicated can it get? *Immunogenetics,* **2010**, *62*(10), 641-651.

[5] Spurkland, A.; Ingvarsson, G.; Falk, E.S.; Knutsen, I.; Sollid, L.M.; Thorsby, E. Dermatitis herpetiformis and celiac disease are both primarily associated with the HLA-DQ (Alpha 1*0501, Beta 1*02) or the HLA-DQ (Alpha 1*03, Beta 1*0302) heterodimers. *Tissue Antigens,* **1997**, *49*(1), 29-34.

[6] Jackson, J.R.; Eaton, W.W.; Cascella, N.G.; Fasano, A.; Kelly, D.L. Neurologic and psychiatric manifestations of celiac disease and gluten sensitivity. *Psychiatr. Q,* **2012**, *83*, 91-102.

[7] Hadjivassiliou, M.; Sanders, D.S.; Woodroofe, N.; Williamson, C.; Grünewald, R.A. Gluten ataxia. *Cerebellum,* **2008**, *7*(3), 494-498.

[8] Hadjivassiliou, M.; Sanders, D.D.; Aeschlimann, D.P. Gluten-related disorders: Gluten ataxia. *Dig. Dis.,* **2015**, *33*(2), 264-268.

[9] Gerace, E.; Resta, F.; Landucci, E.; Renzi, D.; Masi, A.; Pellegrini-Giampietro, D.E.; Calabrò, A.; Mannaioni, G. The gliadin peptide 31-43 exacerbates kainate neurotoxicity in epilepsy models. *Sci. Rep.,* **2017**, *7*(1), 15146.

[10] Drago, S.; El Asmar, R.; Di Pierro, M.; Clemente, M.G.; Tripathi, A.; Sapone, A.; Thakar, M.; Iacono, G.; Carroccio, A.; D'Agate, C.; Not, T.; Zampini, L.; Catassi, C.; Fasano, A. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand. J. Gastroenterol.,* **2006**, *41*(4), 408-419.

[11] Lammers, K.M.; Lu, R.; Brownley, J.; Lu, B.; Gerard, C.; Thomas, K.; Rallabhandi, P.; Shea-Donohue, T.; Tamiz, A.; Alkan, S.; Netzel-Arnett, S.; Antalis, T.; Vogel, S.N.; Fasano, A. Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. *Gastroenterology,* **2008**, *135*(1), 194-204.

[12] Banc, A.; Desbat, B.; Renard, D.; Popineau, Y.; Mangavel, C.; Navailles, L. Exploring the Interactions of gliadins with model

membranes: Effect of confined geometry and interfaces. *Biopolymers,* **2009**, *91*(8), 610-622.
[13] Herrera, M.G.; Benedini, L.A.; Lonez, C.; Schilardi, P.L.; Hellweg, T.; Ruysschaert, J.M.; Dodero, V.I. Self-assembly of 33-mer gliadin peptide oligomers. *Soft Matter,* **2015**, *11*(44), 8648-8660.
[14] Herrera, M.G.; Veuthey, T.V.; Dodero, V.I. Self-organization of gliadin in aqueous media under physiological digestive pHs. *Coll. Surfaces B Biointerfaces* **2016**, *141*, 565-575.
[15] Herrera, M.G.; Zamarreño, F.; Costabel, M.; Ritacco, H.; Hütten, A.; Sewald, N.; Dodero, V.I. Circular dichroism and electron microscopy studies *in vitro* of 33-mer gliadin peptide revealed secondary structure transition and supramolecular organization. *Biopolymers,* **2014**, *101*(1), 96-106.
[16] Fasano, A.; Catassi, C. Clinical practice. Celiac disease. *N. Engl. J. Med.,* **2012**, *367*(25), 2419-2426.
[17] Parzanese, I.; Qehajaj, D.; Patrinicola, F.; Aralica, M.; Chiriva-Internati, M.; Stifter, S.; Elli, L.; Grizzi, F. Celiac disease: From pathophysiology to treatment. *World J. Gastrointest. Pathophysiol.,* **2017**, *8*(2), 27-38.
[18] Panagiotou, S.; Kontogianni, M.D. The economic burden of gluten-free products and gluten-free diet: A cost estimation analysis in Greece. *J. Hum. Nutr. Diet.,* **2017**, *30*(6), 746-752.
[19] Al-Bawardy, B.; Codipilly, D.C.; Rubio-Tapia, A.; Bruining, D.H.; Hansel, S.L.; Murray, J.A. Celiac disease: A clinical review. *Abdom. Radiol. (New York),* **2017**, *42*(2), 351-360.
[20] Niewinski, M.M. Advances in celiac disease and gluten-free diet. *J. Am. Diet. Assoc.,* **2008**, *108*(4), 661-672.
[21] Watkins, R.D.; Zawahir, S. Celiac disease and nonceliac gluten sensitivity. *Pediatr. Clin. North Am.,* **2017**, *64*(3), 563-576.
[22] Matthias, T.; Neidhöfer, S.; Pfeiffer, S.; Prager, K.; Reuter, S.; Gershwin, M.E. Novel trends in celiac disease. *Cell. Mol. Immunol.,* **2011**, *8*(2), 121-125.
[23] Briani, C.; Samaroo, D.; Alaedini, A. Celiac disease: From gluten to autoimmunity. *Autoimmun. Rev.,* **2008**, *7*(8), 644-650.
[24] Schuppan, D.; Zimmer, K.P. The diagnosis and treatment of celiac disease. *Dtsch. Arztebl. Int.,* **2013**, *110*(49), 835-846.
[25] Lionetti, E.; Gatti, S.; Pulvirenti, A.; Catassi, C. Celiac disease from a global perspective. *Best Pract. Res. Clin. Gastroenterol.,* **2015**, *29*(3), 365-379.
[26] Pérez-Diez, C.; Guillén-Lorente, S.; Palomo-Palomo, P. What medication should be prescribed to a patient with coeliac disease?. *Semergen,* **2017**, *44*(2) 121-124.
[27] Gaesser, G.A.; Angadi, S.S. Gluten-free diet: Imprudent dietary advice for the general population? *J. Acad. Nutr. Diet.,* **2012**, *112*(9), 1330-1333.
[28] Green, P.H.R.; Lebwohl, B.; Greywoode, R. Celiac disease. *J. Allergy Clin. Immunol.,* **2015**, *135*(5), 1099-1106.
[29] Villalta, D.; Girolami, D.; Bidoli, E.; Bizzaro, N.; Tampoia, M.; Liguori, M.; Pradella, M.; Tonutti, E.; Tozzoli, R. High prevalence of celiac disease in autoimmune hepatitis detected by anti-tissue tranglutaminase autoantibodies. *J. Clin. Lab. Anal.,* **2005**, *19*(1), 6-10.
[30] Fasano, A.; Berti, I.; Gerarduzzi, T.; Not, T.; Colletti, R. B.; Drago, S.; Elitsur, Y.; Green, P.H.R.; Guandalini, S.; Hill, I.D.; Pietzak, M.; Ventura, A.; Thorpe, M.; Kryszak, D.; Fornaroli, F.; Wasserman, S.S.; Murray, J.A.; Horvath, K. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: A large multicenter study. *Arch. Intern. Med.,* **2003**, *163*(3), 286-292.
[31] Kaur, A.; Shimoni, O.; Wallach, M. Celiac disease: From etiological factors to evolving diagnostic approaches. *J. Gastroenterol.,* **2017**, *52*(9), 1001-1012.
[32] Herrán, A.R.; Pérez-Andrés, J.; Caminero, A.; Nistal, E.; Vivas, S.; Ruiz de Morales, J.M.; Casqueiro, J. Gluten-degrading bacteria are present in the human small intestine of healthy volunteers and celiac patients. *Res. Microbiol.,* **2017**, *168*(7), 673-684.
[33] Reif, S.; Lerner, A. Tissue transglutaminase—the key player in celiac disease: A review. *Autoimmun. Rev.,* **2004**, *3*(1), 40-45.
[34] Fernandez-Feo, M.; Wei, G.; Blumenkranz, G.; Dewhirst, F.E.; Schuppan, D.; Oppenheim, F.G.; Helmerhorst, E.J. The cultivable human oral gluten-degrading microbiome and its potential implications in coeliac disease and gluten sensitivity. *Clin. Microbiol. Infect.,* **2013**, *19*(9), E386-E394.
[35] du Pré, M.F.; Sollid, L.M. T-cell and B-cell immunity in celiac disease. *Best Pract. Res. Clin. Gastroenterol.,* **2015**, *29*(3), 413-423.
[36] Sollid, L.M. The roles of MHC class II genes and post-translational modification in celiac disease. *Immunogenetics,* **2017**, *69*(8-9), 605-616.
[37] Shan, L. Structural basis for gluten intolerance in celiac sprue. *Science,* **2002**, *297*(5590), 2275-2279.
[38] Mcl Mowat, A. Coeliac disease—a meeting point for genetics, immunology, and protein chemistry. *Lancet,* **2003**, *361*(9365), 1290-1292.
[39] Shannahan, S.; Leffler, D.A. Diagnosis and updates in celiac disease. *Gastrointest. Endosc. Clin. N. Am.,* **2017**, *27*(1), 79-92.
[40] Dubé, C.; Rostom, A.; Sy, R.; Cranney, A.; Saloojee, N.; Garritty, C.; Sampson, M.; Zhang, L.; Yazdi, F.; Mamaladze, V.; Pan, I.; Macneil, J.; Mack, D.; Patel, D.; Moher, D. The prevalence of celiac disease in average-risk and at-risk western European populations: A systematic review. *Gastroenterology,* **2005**, *128*(4), S57-S67.
[41] Greco, L. The first large population based twin study of coeliac disease. *Gut,* **2002**, *50*(5), 624.
[42] Tollefsen, S.; Arentz-Hansen, H.; Fleckenstein, B.; Molberg, Ø.; Ráki, M.; Kwok, W.; Jung, G.; Kea, L.; Sollid, L. HLA-DQ2 and -DQ8 signatures of gluten t cell epitopes in celiac disease. *J. Clin. Invest.,* **2006**, *116*(8), 2226-2236.
[43] Stamnaes, J.; Sollid, L.M. Celiac disease: Autoimmunity in response to food antigen. *Semin. Immunol.,* **2015**, *27*(5), 343-352.
[44] McAllister, C.S.; Kagnoff, M.F. The immunopathogenesis of celiac disease reveals possible therapies beyond the gluten-free diet. *Semin. Immunopathol.,* **2012**, *34*(4), 581-600.
[45] Lebwohl, B.; Sanders, D.S.; Green, P.H.R. Coeliac disease. *Lancet,* **2018**, *391*(10115), 70-81.
[46] Chmielewska, A.; Piescik-Lech, M.; Szajewska, H.; Shamir, R. Primary prevention of celiac disease: environmental factors with a focus on early nutrition. *Ann. Nutr. Metab.,* **2015**, *67*(2), 43-50.
[47] Aronsson, C.A.; Lee, H.S.; Liu, E.; Uusitalo, U.; Hummel, S.; Yang, J.; Hummel, M.; Rewers, M.; She, J.X.; Simell, O.; Toppari, J.; Ziegler, A.G.; Krischer, J.; Virtanen, S.M.; Norris, J.M.; Agardh, D.; TEDDY STUDY GROUP. Age at gluten introduction and risk of celiac disease. *Pediatrics,* **2015**, *135*(2), 239-245.
[48] Szajewska, H.; Shamir, R.; Mearin, L.; Ribes-Koninckx, C.; Catassi, C.; Domellöf, M.; Fewtrell, M.S.; Husby, S.; Papadopoulou, A.; Vandenplas, Y.; Castillejo, G.; Kolacek, S.; Koletzko, S.; Korponay-Szabó, I.R.; Lionetti, E.; Polanco, I.; Troncone, R. Gluten introduction and the risk of coeliac disease. *J. Pediatr. Gastroenterol. Nutr.,* **2016**, *62*(3), 507-513.
[49] Stene, L.C.; Honeyman, M.C.; Hoffenberg, E.J.; Haas, J.E.; Sokol, R.J.; Emery, L.; Taki, I.; Norris, J.M.; Erlich, H.A.; Eisenbarth, G.S.; Rewers, M. Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: A longitudinal study. *Am. J. Gastroenterol.,* **2006**, *101*(10), 2333-2340.
[50] Riddle, M.S.; Murray, J.A.; Porter, C.K. The incidence and risk of celiac disease in a healthy US adult population. *Am. J. Gastroenterol.,* **2012**, *107*(8), 1248-1255.
[51] Lebwohl, B.; Ludvigsson, J.F.; Green, P.H.R. Celiac disease and non-celiac gluten sensitivity. *Br. Med. J. (Clin. Res. Ed).,* **2015**, *351*, 4347.
[52] Nijhawan, S.; Goyal, G. Celiac disease review. *J. Gastrointest. Dig. Syst.,* **2015**, *5*(6), 350.
[53] Ludvigsson, J.F.; Leffler, D.A.; Bai, J.C.; Biagi, F.; Fasano, A.; Green, P.H.R.; Hadjivassiliou, M.; Kaukinen, K.; Kelly, C.P.; Leonard, J.N.; Lundin, K.E.; Murray, J.A.; Sanders, D.S.; Walker, M.M.; Zingone, F.; Ciacci, C. The oslo definitions for coeliac disease and related terms. *Gut,* **2013**, *62*(1), 43-52.
[54] Fasano, A. Clinical presentation of celiac disease in the pediatric population. *Gastroenterology,* **2005**, *128*(4), S68-S73.
[55] Green, P.H.R.; Krishnareddy, S.; Lebwohl, B. Clinical manifestations of celiac disease. *Dig. Dis.,* **2015**, *33*(2), 137-140.
[56] Green, P.H.R. The many faces of celiac disease: Clinical presentation of celiac disease in the adult population. *Gastroenterology,* **2005**, *128*(4), S74-S78.
[57] Briani, C.; Zara, G.; Alaedini, A.; Grassivaro, F.; Ruggero, S.; Toffanin, E.; Paola, M.; Luca, M.; Giometto, B.; Ermani, M.; De Lazzari, F.; D'Odorico, A.; Battistin, L. Neurological complications of celiac disease and autoimmune mechanisms: A prospective study. *J. Neuroimmunol.,* **2008**, *195*, 171-175.
[58] Catassi, C.; Fabiani, E.; Iacono, G.; D'Agate, C.; Francavilla, R.; Biagi, F.; Volta, U.; Accomando, S.; Picarelli, A.; De Vitis, I.; Pianelli, G.; Gesuita, R.; Carle, F.; Mandolesi, A.; Bearzi, I.;

Fasano, A. A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *Am. J. Clin. Nutr.,* **2007**, *85*(1), 160-166.

[59] Jericho, H.; Sansotta, N.; Guandalini, S. Extraintestinal manifestations of celiac disease: Effectiveness of the gluten-free diet. *J. Pediatr. Gastroenterol. Nutr.,* **2017**, *65*(1), 75-79.

[60] Hall, N.; Rubin, G.; Charnock, A. Systematic review: Adherence to a gluten-free diet in adult patients with coeliac disease. *Aliment. Pharmacol. Ther.,* **2009**, *30*(4), 315-330.

[61] Gobbetti, M.; Pontonio, E.; Filannino, P.; Rizzello, C.G.; De Angelis, M.; Di Cagno, R. How to improve the gluten-free diet: The state of the art from a food science perspective. *Food Res. Int.,* **2017 (in press)**.

[62] Estévez, V.; Ayala, J.; Vespa, C.; Araya, M. The gluten-free basic food basket: A problem of availability, cost and nutritional composition. *Eur. J. Clin. Nutr.,* **2016**, *70*(10), 1215-1217.

[63] Lambert, K.; Ficken, C. Cost and affordability of a nutritionally balanced gluten-free diet: Is following a gluten-free diet affordable? *Nutr. Diet.,* **2016**, *73*(1), 36-42.

[64] Foschia, M.; Horstmann, S.; Arendt, E.K.; Zannini, E. Nutritional therapy – facing the gap between coeliac disease and gluten-free food. *Int. J. Food Microbiol.,* **2016**, *239*, 113-124.

[65] Bernardo, D.; Garrote, J.A.; Nadal, I.; Leon, A.J.; Calvo, C.; Fernandez-Salazar, L.; Blanco-Quiros, A.; Sanz, Y.; Arranz, E. Is it true that coeliacs do not digest gliadin? degradation pattern of gliadin in coeliac disease small intestinal mucosa. *Gut,* **2009**, *58*(6), 886-887.

[66] Case, S. The Gluten-free diet: How to provide effective education and resources. *Gastroenterology,* **2005**, *128*(4), S128-S134.

[67] Vici, G.; Belli, L.; Biondi, M.; Polzonetti, V. Gluten free diet and nutrient deficiencies: A review. *Clin. Nutr,.* **2016**, *35*(6), 1236-1241.

[68] Caminero, A.; Galipeau, H.J.; McCarville, J.L.; Johnston, C.W.; Bernier, S.P.; Russell, A.K.; Jury, J.; Herran, A.R.; Casqueiro, J.; Tye-Din, J.A.; Surette, M.G.; Magarvey, N.A.; Schuppan, D.; Verdu, E.F. Duodenal bacteria from patients with celiac disease and healthy subjects distinctly affect gluten breakdown and immunogenicity. *Gastroenterology,* **2016**, *151*(4), 670-683.

[69] Kim, S.M.; Mayassi, T.; Jabri, B. Innate immunity: Actuating the gears of celiac disease pathogenesis. *Best Pract. Res. Clin. Gastroenterol.,* **2015**, *29*(3), 425-435.

[70] Caminero, A.; Herrán, A.R.; Nistal, E.; Pérez-Andrés, J.; Vaquero, L.; Vivas, S.; Ruiz de Morales, J.M.G.; Albillos, S.M.; Casqueiro, J. Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease. *FEMS Microbiol. Ecol.,* **2014**, *88*(2), 309-319.

[71] Siegel, M.; Garber, M.E.; Spencer, A.G.; Botwick, W.; Kumar, P.; Williams, R.N.; Kozuka, K.; Shreeniwas, R.; Pratha, V.; Adelman, D.C. Safety, tolerability, and activity of ALV003: Results from two phase 1 single, escalating-dose clinical trials. *Dig. Dis. Sci.,* **2012**, *57*(2), 440-450.

[72] Shan, L.; Molberg, Ø.; Parrot, I.; Hausch, F.; Filiz, F.; Gray, G.M.; Sollid, L.M.; Khosla, C. Structural basis for gluten intolerance in celiac sprue. *Science,* **2002**, *297*(5590), 2275-2279.

[73] Hausch, F.; Shan, L.; Santiago, N.A.; Gray, G.M.; Khosla, C. Intestinal digestive resistance of immunodominant gliadin peptides. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **2002**, *283*(4), G996-G1003.

[74] Pandey, V.; Solanki, N.; Berwal, V.; Malik, N. Probiotics: Healthy bugs and nourishing elements of diet. *J. Int. Soc. Prev. Community Dent.,* **2015**, *5*(2), 81-87.

[75] Lorenzo Pisarello, M.J.; Vintiñi, E.O.; González, S.N.; Pagani, F.; Medina, M.S. Decrease in lactobacilli in the intestinal microbiota of celiac children with a gluten-free diet, and selection of potentially probiotic strains. *Can. J. Microbiol.,* **2015**, *61*(1), 32-37.

[76] De Palma, G.; Nadal, I.; Collado, M.C.; Sanz, Y. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. *Br. J. Nutr.,* **2009**, *102*(8), 1154-1160.

[77] Goel, G.; King, T.; Daveson, A.J.; Andrews, J.M.; Krishnarajah, J.; Krause, R.; Brown, G.J.E.; Fogel, R.; Barish, C.F.; Epstein, R.; Kinney, T.P.; Miner, P.B.; Jr, Tye-Din, J.A.; Girardin, A.; Taavela, J.; Popp, A.; Sidney, J.; Mäki, M.; Goldstein, K.E.; Griffin, P.H.; Wang, S.; Dzuris, J.L.; Williams, L.J.; Sette, A.; Xavier, R.J.; Sollid, L.M.; Jabri, B.; Anderson, R.P. Epitope-specific immunotherapy targeting CD4-positive T cells in coeliac disease: Two randomised, double-blind, placebo-controlled phase 1 studies. *Lancet Gastroenterol. Hepatol.,* **2017**, *2*(7), 479-493.

[78] Di Sabatino, A.; Corazza, G.R. Epitope-specific immunotherapy: A vaccine for coeliac disease? *Lancet Gastroenterol. Hepatol.,* **2017**, *2*(7), 464-465.

[79] Wieser, H. Chemistry of gluten proteins. *Food Microbiol.,* **2007**, *24*(2), 115-119.

[80] Wieser, H. Relation between gliadin structure and coeliac toxicity. *Acta Paediatr. Suppl.,* **1996**, *412*(1), 3-9.

[81] Shewry, P.R.; Tatham, A.S. Disulphide bonds in wheat gluten proteins. *J. Cereal Sci.,* **1997**, *25*(3), 207-227.

[82] Wieser, H.; Kieffer, R. Correlations of the amount of gluten protein types to the technological properties of wheat flours determined on a micro-scale. *J. Cereal Sci.,* **2001**, *34*(1), 19-27.

[83] Grosch, W.; Wieser, H. Redox reactions in wheat dough as affected by ascorbic acid. *J. Cereal Sci.,* **1999**, *29*(1), 1-16.

[84] Cornell, H.J.; Maxwell, R.J. Amino acid composition of gliadin fractions which may be toxic to individuals with coeliac disease. *Clin. Chim. Acta,* **1982**, *123*(3), 311-319.

[85] Kasarda, D.D.; Bernardin, J.E.; Thomas, R.S. Reversible aggregation of alpha-gliadin to fibrils. *Science,* **1967**, *155*(3759), 203-205.

[86] Field, J.M.; Shewry, P.R.; Miflin, B.J. Aggregation states of alcohol-soluble storage proteins of barley, rye, wheat and maize. *J. Sci. Food Agric.,* **1983**, *34*(4), 362.

[87] Cole, E.; Torres, J.; Kasarda, D.D. Aggregation of a-gliadin: Gel permeation chromatography. *Cereal Chem.,* **1983**, *60*, 306-310.

[88] Amin, S.; Barnett, G.V.; Pathak, J.A.; Roberts, C.J.; Sarangapani, P.S. Protein aggregation, particle formation, characterization & rheology. *Curr. Opin. Colloid Interface Sci.,* **2014**, *19*(5), 438-449.

[89] Philo, J.S.; Arakawa, T. Mechanisms of protein aggregation. *Curr. Pharm. Biotechnol.,* **2009**, *10*(4), 348-351.

[90] Pekar, A.H.; Frank, B.H. Conformation of proinsulin. A comparison of insulin and proinsulin self-association at neutral pH. *Biochemistry,* **1972**, *11*(22), 4013-4016.

[91] Quirolo, Z.B.; Benedini, L.A.; Sequeira, M.A.; Herrera, M.G.; Veuthey, T.V.; Dodero, V.I. Understanding recognition and self-assembly in biology using the chemist's toolbox. insight into medicinal chemistry. *Curr. Top. Med. Chem.,* **2014**, *14*(6), 730-739.

[92] Krishnamurthy, R.; Manning, M.C. The stability factor: Importance in formulation development. *Curr. Pharm. Biotechnol.,* **2002**, *3*(4), 361-371.

[93] Lehn, J.M. Toward complex matter: supramolecular chemistry and self-organization. *Proc. Natl. Acad. Sci. USA,* **2002**, *99*(8), 4763-4768.

[94] Horwich, A. Protein aggregation in disease: A role for folding intermediates forming specific multimeric interactions. *J. Clin. Invest.,* **2002**, *110*(9), 1221-1232.

[95] Hartgerink, J.D. Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science,* **2001**, *294*(5547), 1684-1688.

[96] Calderdn de la Barca, A.; Yepiz-Plascencia, M.G.M.T.C.B.H. Hydrophobic interactions between gliadin and proteins and celiac disease. *Life Sci.,* **1996**, *59*(23), 19511960.

[97] Garcia-Quintanilla, A.; Miranzo-Navarro, D. Extraintestinal manifestations of celiac disease: 33-mer gliadin binding to glutamate receptor GRINA as a new explanation. *BioEssays,* **2016**, *38*(5), 427-439.

[98] Wüthrich, K. The way to NMR structures of proteins. *Nat. Struct. Biol.,* **2001**, *8*(11), 923-925.

[99] Torrent, J.; Alvarez-martinez, M.T.; Liautard, J.; Balny, C.; Lange, R. The role of the 132 – 160 region in prion protein conformational transitions. *Prot. Sci.,* **2005**, *14*, 956-967.

[100] Esfandiary, R.; Middaugh, C.R. Ultraviolet Absorption Spectroscopy. In *Analysis of Aggregates and Particles in Protein Pharmaceuticals*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2012; pp. 169-200.

[101] Dodero, V.I.; Messina, P.V. Chapter 4: Analyzing the Solution State of Protein Structure, Interactions, and Ligands by Spectroscopic Methods. In *Proteins in solution and at Interfases*; Ruso, J.M.; Piñeiro, A.; Eds.; John Wiley & Sons, Inc.: New York, 2013; pp. 73-98.

[102] Kasarda, D.D.; Bernardin, J.E.; Gaffield, W. Circular dichroism and optical rotatory dispersion of alpha-gliadin. *Biochemistry,* **1968**, *7*(11), 3950-3957.

[103] Royer, C.A. Probing protein folding and conformational transitions with fluorescence probing protein folding and conformational transitions with fluorescence. **2006**, *106*, 1769-1784.

[104] Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*, 3rd edition; Lakowicz, J.R.; Ed.; Springer US: Boston, MA, **2006**.

[105] Jones, A.R. Light scattering for particle characterization. *Prog. Energy Combust. Sci.,* **1999**, *25*, 1-53.

[106] Berne, B.J.; Pecora, R. Dynamic Light Scattering: With Applications to Chemistry, Biology, and Physics; John Wiley & Sons, Inc., New York, **2000**.

[107] Carpenter, D.K. Dynamic light scattering with applications to chemistry, biology, and physics (Berne, Bruce J.; Pecora, Robert). *J. Chem. Educ.,* **1977**, *54*(10), A430.

PMID: 29852868