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Living with celiac disease beyond the diagnosis: food safety, adherence to diet, quality of life and the emerging role of wheat amylase-trypsin inhibitors as potent triggers of innate intestinal immunity

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Chapter 1 **– INTRODUCTION**

1.1. Gluten related disorders

Wheat, rice and maize are the most widely consumed food cereals worldwide. More than 25,000 different cultivars have been produced by wheat breeding, with much of the production consumed after processing into bread, pasta, noodles, and, in the Middle East and North Africa, bulgur and couscous. The wide availability of wheat flour and the functional properties of gluten proteins contributed to their wide use as an ingredient in food processing. Gluten is also found in other cereals like oat, rye, and barley. Wheat grain include three major components: starch, proteins, and cell wall polysaccharides, accounting for about 90% of the dry weight. Gluten proteins represents about 80% of the total protein content in grain. Gluten is not a single protein but a mixture indicated as prolamins. Gluten proteins are divided into two major fractions: (i) Gliadins (monomeric, subdivided into ω-, γ-, and α/β-gliadin fractions), and (ii) Glutenins (large polymers). In particular, the most immunogenic gluten fragment is the 33-mer peptide, which consists of 33 amino acids of the α-gliadin fraction. Several diseases and digestive disorders are ascribed to gluten consumption [1-3]. The spectrum of gluten-related disorders includes conditions such as celiac disease (CD), non-celiac gluten sensitivity (NCGS), dermatitis herpetiformis, gluten ataxia, and wheat allergy (WA), in which the immune system reacts to and deals with the triggering environmental factor, e.g. gliadin, in distinct ways [2]. In order to develop a consensus on nomenclature and classification of gluten-related disorders, a panel of 15 experts convened in London in 2011 [2]. The high frequency and wide range of adverse reactions to gluten raised during the last 50 years can find three possible explanations: (i) the selection of wheat varieties with higher gluten content, with changes dictated more by technological rather than nutritional reasons, as particularly suitable for intensive agriculture; (ii) greater awareness and knowledge of the disease by the medical profession; (iii) the availability of more sensitive, specific and less invasive diagnostic tests able to detect patients who until a few years ago would have remained undiagnosed. In

Europe, the mean daily gluten consumption of gluten is about 10-20 g, with exceptions consuming up to 50 g or more.

1.1.1. Celiac disease

CD is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in genetically predisposed individuals [3]. It is one of the most frequent lifelong disorders, affecting approximately 1-2% of the general population worldwide and a considerable number of undiagnosed patients [2]. The presence of the human leukocyte antigen (HLA)-DQ2 and/or -DQ8 haplotypes, identified in the HLA class II histocompatibility system expressed on the surface of antigen-presenting cells, mainly macrophages, dendritic cells, and B cells, is considered as a necessary risk factor for the development of CD. Risk of CD is excluded if both genotypes are absent, with nearly 95% confidence [4]. The current diagnostic algorithm for CD includes initial screening serological tests, in particular the combination of total serum IgA and IgA-antibodies against transglutaminase (TGA-IgA), and, only if total IgA is low/undetectable, an IgG-based test. Patients with positive results should be referred to a specialist gastroenterologist. If TGA-IgA is ≥10 times the upper normal limit (10× ULN) and the family agrees, the no-biopsy diagnosis can be applied, provided symptoms suggestive of CD (particularly malabsorption) and positive IgA-endomysial antibodies (EMA-IgA), because enteropathy (Marsh 2 or 3) is nearly always present in patients with very high coeliac auto-antibody levels in serum. HLA DQ2-/DQ8 determination is not mandatory criteria. Children with positive TGA-IgA but lower titers (<10 times upper limit of normal) should undergo at least 4 biopsies from the distal duodenum and at least 1 from the bulb to decrease the risk of false positive diagnosis. Discordant results between serology and histopathology may require a second evaluation of biopsies. Patients with no/mild histological changes (Marsh 0/1) but confirmed autoimmunity (TGA-IgA/EMA-IgA positivity) are advised to follow strict follow-up schedules [5]. In CD, both innate and adaptive immunity are involved. Gluten peptides pass through the intestinal epithelium and, once in the lamina propria, the immunogenicity is markedly increased after their deamidation by tissue transglutaminases. This will enhance the

presentation of dendritic cells to T-lymphocyte cells, which produce abundant amounts of pro-inflammatory cytokines and increases the cytotoxicity of intraepithelial lymphocytes. The villous atrophy in the intestinal mucosa of CD patients is caused by enterocytic apoptosis. The intestinal damage is a slow and gradual process and the mechanism that causes the severity of clinical symptomatology is still unknown. So far, a non-invasive molecular biomarker able to identify the development of mucosal lesions is still lacking. Several studies demonstrated that the degree of villous atrophy does not correlate with clinical manifestations. Without changing eating habits, a persistent intestinal villous atrophy would lead to the inability to sufficiently metabolize nutrients, resulting in malnutrition. In addition, it appears that the microbiota varies both in treated and untreated CD subjects, compared to healthy controls. In particular, Firmicutes and Bifidobacteria appear to decrease, while Proteobacteria, Bacteroides, and E. coli increase [6]. However, whether these changes are a cause or consequence of CD it is still unknown, i.e. the gluten-free diet (GFD) is often low in fibre, and this could influence the composition of the microbiota. The clinical spectrum of CD is wide and include the so-called classical form (including symptoms and signs of malabsorption such as diarrhea, weight and energy loss, iron deficiency with anemia, deficiency of vitamins and/or minerals and malnutrition), the non-classic form (with predominant extraintestinal manifestations) e the silent form (patients do not complain any symptoms, but still experience villous atrophy). In addition, potential CD represents around 10% of the diagnoses, in most cases asymptomatic children belonging to risk categories (positive serological markers of CD, positive genetic test HLA-DQ2 and/or -DQ8, and normal small intestinal mucosa). The onset of symptoms is usually gradual and characterized by a time lag of months or years after gluten introduction. Even very small amounts of gluten can cause health problems. Complications of CD are rare, and the most frequent include refractory CD type 1 and type 2 (lack of clinical and histological response to at least 12-15 months of a rigorous GFD or reappearance of villous atrophy despite a strictly followed GFD), ulcerative jejuno-ileitis, enteropathy T-cell type 1 lymphoma, intra-abdominal B cell lymphoma, and carcinoma of the small intestine. Patients who do not follow a strict GFD are at an increased risk of developing complications. Among

patients following a strict GFD, the risk is enhanced in subjects diagnosed after 40 years old and for those with symptoms of major malabsorption. Patients at high risk of complications should be monitored every 6-12 months with clinical evaluation and blood biochemistry examinations. In children, CD develops during the first years of life, after weaning when they switch from breast milk to gluten containing foods. A late diagnosis can lead to growth and development disorders. So far, a strict and permanent GFD is the only effective treatment. The GFD determines disappearance of celiac-related symptoms and serum autoantibodies, recovery of intestinal mucosa, and prevention of long-term complications [7]. However, in patients on long-term treatment with a GFD, the ingestion of gluten may occasionally cause immediate symptoms, such as vomiting and abdominal pain.

1.1.2. Wheat allergy

Wheat allergy (WA) is defined as an adverse immunologic reaction to wheat proteins occurring minutes or hours after wheat exposure. It is classified into: (i) classic food allergy triggered by wheat proteins ingestion; (ii) wheat dependent exercise-induced anaphylaxis (WDEIA); (iii) occupational asthma (baker's asthma) and rhinitis; (iv) contact urticaria. IgE antibodies play a key role in the pathogenesis of WA. The serological markers of CD are negative, there is no correlation with HLA-DQ2/DQ8 haplotypes, and the intestinal mucosa is normal. The prevalence of WA is relatively low (0.25%). Dietary allergy due to wheat ingestion may lead to anaphylaxis and death. Baker's asthma and rhinitis are well-characterized allergic responses to wheat flours and dusts inhalation. WDEIA is a well-defined syndrome that is caused by a specific type of grain protein, ω5-gliadins. Other allergic responses such as atopic dermatitis, urticaria and anaphylaxis seem to be related to a range of proteins in wheat. First-level diagnostics for WA are skin prick tests and in vitro IgE assays and many cases require an oral food challenge for the final diagnosis [2].

1.1.3. Non-celiac gluten sensitivity

The pathogenesis of non-celiac gluten sensitivity (NCGS) is still unclear. It should be considered in all patients with persistent intestinal and/or extraintestinal symptoms occurring in a few hours or days after ingestion of gluten/wheat-containing food, negative serological markers of CD and WA while on a gluten-containing diet, reporting worsening of symptoms after eating gluten-rich food [3]. The first cases of NCGS were reported in the 1970s but only recently it has been characterized as a disorder clinically distinct from CD [2]. The pathogenesis on NCGS is likely to be multifactorial, with the innate immune response playing a key role. The definition of NCGS has been recently discussed at four consensus conferences [2-4, 7]. According to the expert panel, NCGS should be defined as a non-allergic and non-autoimmune condition, with intestinal and/or extraintestinal symptoms caused by gluten containing food, after exclusion of both CD and WA [4]. Due to lack of a disease biomarker, the prevalence of NCGS is still unclear. Current estimates indicate a prevalence of around 2% in the general population and 0.2-0.3% in children [8, 9]. The classical presentation of NCGS is a combination of irritable bowel syndrome (IBS)-like symptoms, including abdominal pain, bloating, bowel habit abnormalities (either diarrhea or constipation), and minimal neurological manifestations such as ‘foggy mind', headache, chronic fatigue, joint and muscle pain, leg or arm numbness, dermatitis (eczema or skin rash), depression, and anemia [3]. In recent years, several studies explored the relationship between the ingestion of gluten-containing food and the appearance of neurological and psychiatric disorders/symptoms such as ataxia, peripheral neuropathy, schizophrenia, autism, depression, anxiety, and hallucinations (so-called gluten psychosis) [3]. It has been hypothesized that symptoms may be caused by opioid peptides derived from the incomplete breakdown of foods containing gluten and casein. Despite its popularity, the efficacy of the gluten-free-casein-free (GFCF) diet in improving autistic behavior remains to be proven by high quality randomized controlled trials. In NCGS, specific CD autoantibodies are absent, there is no association with HLA DQ2/DQ8 haplotypes, normal intestinal mucosa and frequent IgG-class antigliadin antibodies (AGA) against native gliadin

(the first-generation AGA test) positivity [3]. However, the lack of a sufficiently sensitive and specific biomarker for diagnostic purposes, allow only a diagnosis based on the exclusion of other gluten-related disorders. In 2014, a group of world experts on gluten-related disorders defined the so-called “Salerno diagnostic criteria”, the diagnostic algorithm of NCGS. It is based on establishing a clear-cut cause-effect relationship between the ingestion of wheat/gluten and the appearance of symptoms [4]. After a full clinical and laboratory evaluation to exclude CD and WA, the protocol includes a two-stage dietary process using a modified version of Gastrointestinal Symptom Rating Scale to evaluate intestinal and extraintestinal symptoms. During phase 1, the patient will follow a gluten-containing diet for 6 weeks and then switch to a GFD for 6 weeks. Patients showing >30% reduction of one to three main symptoms or at least 1 symptom with no worsening of others) for at least 50% of the observation time will be defined responders and will undergo the phase 2. Due to the high rate of perceived gluten sensitivity and the possible placebo/nocebo effect of any dietary intervention, a double blind, placebo-controlled gluten challenge represents a crucial step. The gluten challenge includes a 1-week challenge followed by a 1-week washout on a strict GFD and then the crossover to the second 1-week challenge. The duration of the challenge period may occasionally be longer than 1 week in patients with fluctuating symptoms. To discriminate a positive from a negative result, a variation of at least 30% between the gluten and the placebo challenge should be observed. GFD-unresponsive patients should be investigated for other possible causes of IBS-like symptoms, e.g., intolerance to FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols) or small bowel bacterial overgrowth. It was previously assumed that gluten was the only wheat component responsible of triggering this disorder. Several studies suggested that wheat components other than gluten, particularly amylase-trypsin inhibitors (ATI) and the so-called FODMAPs, may elicit symptoms of NCGS [3,10-12]. ATI-induced *in vivo* trypsin inhibition may result in increased levels of non-digested bioactive wheat proteins, which may trigger immune responses and inflammation. FODMAPs are fermentable carbohydrates poorly absorbed in the small intestine that might exacerbate intestinal symptoms in sensitive individuals due to gas produced by

fermentation. The terminology of NCGS is still a matter of debate, and since it is often impossible to establish which wheat component/s is/are the disease trigger/s, it could be better defined as NCWS (Non Celiac Wheat Sensitivity). The major limitation of NCWS terminology is the exclusion of other gluten-containing grains, such as rye and barley, which might trigger the disorder.

1.2. Amylase/trypsin inhibitors (ATI)

ATI are low molecular weight wheat proteins, water-soluble, highly disulfide-linked, largely resistant to intestinal proteases and heat. At least 19 ATI isoforms have been described and classified into four groups [12]. The first group includes monomeric inhibitors with the major form named 0.28 (based on electrophoretic mobility), the second group includes the two homodimeric inhibitors called 0.19 and 0.53. The third group includes heterotetrameric inhibitors, which were originally defined as CM proteins (CM1, CM2, CM3, CM16, and CM17). Finally, the fourth group includes inhibitors of trypsin termed CMX. ATI are able to inhibit amylases and/or proteases of different origins, playing an important role in protecting the cereal against pests and parasites. Cereal grains are attractive to pests and pathogens because they have high contents of storage reserves (starch and protein). ATI accumulate in large amounts in the endosperm of wheat and other cereals like barley and rye, with additional functions as reserve proteins. Wheat proteins can be classified in albumins, globulins, gliadins and glutenins, according to their structural properties and solubility. ATI belong to the albumin/globulin fraction, which account for 2–4% of the total wheat protein (as compared to 80–90% for gluten) with important differences in the content and type of ATI between the different genotypic and phenotypic varieties of wheat.

1.2.1. Genetic and environmental impact on ATI in wheat

Common hexaploid wheat (*Triticum aestivum* L. ssp. *aestivum*) comprises three genomes (A, B, and D) derived from ancestor species. The tetraploid species share A and B genome with

common wheat and include emmer (*Triticum turgidum* L. ssp. *dicoccum*), durum (*T. turgidum* L. ssp. *durum*), rivet (*T. turgidum* L. ssp. *turgidum*), and khorasan wheat (*T. turgidum* L. ssp. *turanicum*), while the oldest cultivated wheat is einkorn (*Triticum monococcum* L. ssp. *monococcum*), is diploid and comprises only A genome. All ATI are encoded by B and D genomes, indicating that ancient wheat like eikorn may produce less ATI than modern wheat and therefore have less pro-inflammatory biological activity. [10-12] The individual ATI are encoded by single genes but the total ATI concentration shows polygenic inheritance due to the high number of isoforms. Several studies showed significant impact of genotype, harvest year, environment (i.e. precipitation), and interactions among these factors on inhibitory activity against amylases and trypsin [14,15].

1.2.2. Role of ATI in the development of gluten related disorders (GRD)

In vitro and animal studies suggested that ATI may play a key role in the etiology of CD and other gluten related disorders by eliciting the activation of the adaptive and innate immune systems, disruption of the intestinal barrier function and intestinal/extra-intestinal inflammation [16]. However, only limited information is available about the immunogenic sequences of ATI. [17] An in vitro study by Junker et al. showed that ATI, particularly CM3 and 0.19, induce the innate immune response and activate monocytes, macrophages, and dendritic cells via the TLR4-MD2-CD14 complex with a subsequent release of pro-inflammatory cytokines [16]. Caminero et al. demonstrated that the administration of ATI enriched preparations to nonobese diabetic mice over 2 weeks triggered intestinal intraepithelial lymphocytosis and barrier dysfunction in the absence of overt inflammation or mucosal damage. In addition, the presence of ATI in the large intestine was reported to modify microbiota composition and metabolism [6]. Pickert et al. suggested that ATI associated dysbiosis and ATI-induced TLR4 activation are likely to occur simultaneously and may synergistically promote the overall inflammatory reaction and intestinal barrier function [18]. The suggested mechanism is that ATI may reduce the digestion of gliadins, leading to higher levels of digestion-resistant immunogenic gliadin peptides passing the small intestinal gut epithelium, thus potentiating the

initiation of CD by enhancing the release of pro-inflammatory cytokines and chemokines [19,20]. ATI appear to be also the most potent activators of allergic airway responses, such as bakers' asthma, while they do not appear to trigger the most severe allergic response to wheat consumption, WDEIA, which is triggered by gluten proteins [21,22]. To date, no controlled interventions have been carried out in humans with well-characterized purified compounds isolated from processed wheat-containing foods. In vivo ATI-induced inhibition of trypsin may result in increased levels of non-digested bioactive wheat proteins, which, in turn, may trigger immune responses and inflammation, but at present, there is no clear evidence for this.

1.2.3. Manipulating the ATI content in grain

It is well known that dietary exclusion of wheat-based foods may result in depletion of essential components such as fiber, proteins and minerals. To avoid such exclusion, plant breeding strategies can be used to remove ATI from grain or processing strategies to inactivate them in foods. Two main approaches can be performed to reduce the amount or activity of ATI in plants: (i) to exploit the genetic variation in the content and composition of ATI in different wheat species; (ii) to use mutagenesis or gene editing to reduce the amount and activity of ATI. So far, transgenic lines of wheat bread silenced for CM3, CM16, and 0.28 ATI genes were produced using RNA-interference and genome editing [23, 24].

1.3. Gluten-free diet and patient monitoring

Gluten is the main structural protein complex in wheat with equivalent toxic proteins in other cereals, including rye, barley, spelt, einkorn, khorasan wheat (the most popular marketed as Kamut®), triticale, and oats. The toxic protein fractions of gluten include gliadins and glutenins, with gliadins containing monomeric proteins and glutenins containing aggregated proteins. A strict and permanent GFD is the only effective treatment for CD. The GFD determines disappearance of celiac-related symptoms and serum autoantibodies, recovery of intestinal

mucosa, and prevention of long-term complications [25]. Both children and adults with CD are highly sensitive to the toxic effects of gluten exposure. It has been shown that the protracted ingestion of gluten traces (>10 mg/day) is sufficient to cause significant damage in the architecture of the small intestinal mucosa in patients on treatment for CD. Based on this threshold, a maximum tolerable amount of gluten of <20 parts per million (ppm) in gluten-free food has been calculated [7], a limit that has been endorsed by the major international regulatory agencies, e.g., the Codex Alimentarius, the US Food and Drug Administration (FDA), and the European Food Safety Authority (EFSA) [26-28]. Hence, it is very important to monitor GFD adherence of patients with CD. Dietary interview, clinical symptoms monitoring, CD serology, and small intestinal histology are significant choices; however, they provide only limited and indirect evidence of GFD adherence [29-31]. Moreover, these tools are inadequately sensitive to detect the accidental exposure to traces of dietary gluten. Novel qualitative and quantitative immunochromatographic tests have been developed to directly detect recent dietary exposure to gluten by determining the excretion of gluten immunogenic peptides (GIP) in stools or urine [32-34]. A growing interest has recently focused on the role of stool/urinary GIP determination in the follow-up of treated patients with CD, and this noninvasive and easy to perform test seems to be the most promising and reliable marker of dietary gluten transgressions [35]. However, inadequate information is available about the relationship between the amount of ingested gluten and the quantity of GIP excreted in urine or stool in CD patients, particularly at a low level of gluten ingestion (as is usually the case in treated patients with CD). Unlike food allergies, CD may not cause immediate and severe symptoms after gluten ingestion. This is why patients following GFD, mostly young and asymptomatic or with mild symptoms when diagnosed, tend to occasionally ingest small amounts of gluten because apparently nothing serious happens. Although one single intake of gluten does not trigger any symptoms, the repeated gluten exposure leads to intestinal damage, reappearance of CD specific autoantibodies in blood, increased risk of long-term complications. A prospective, double-blind, placebo-controlled trial by Catassi et al. demonstrated the fundamental importance of maintaining a rigorous GFD to prevent intestinal

damage and potential complications [7]. Upon diagnosis, the guidance of an expert dietician is important to provide education on the GFD, appropriate for age, cultural and social habits of the patient. Adherence to the GFD can benefit from continuous support over time, and a check within 6-12 months after diagnosis and every 1-2 years thereafter (unless complications) is important to verify adequate compliance. CD patients should periodically undergo a medical examination, dietary assessment, IgA class assay of serum antibodies (or IgG if an IgA deficiency is present) and TSH.

1.4. Quality of life in CD patients

As a chronic health condition, CD is defined as an ongoing medical problem worldwide, affecting a growing number of people throughout life. Adherence to the GFD among children and adolescents with CD involves unique challenges in their daily activities and participation. Participation in activities and occupations that are meaningful to the person has direct and substantial impact on health and quality of life. Nowadays, there is a vast gap between the medical treatment guidelines and application of the treatment and self-management in everyday life among children and adolescents with CD. In response to this gap, the children's activities report (CD-Chart) was developed in Israel and administered to 126 children and adolescent aged 8-18 years [36]. The CD-Chart acquires self-perspectives about daily participation in food-related activities. CD research concerning pediatric health-related quality of life (HRQOL) has received increased interested in recent years [37-39]. Although significant associations between HRQOL and adherence have been reported, findings are inconsistent [40]. Primary evidence suggests there are relationships between specific cognitive functions required for daily-self-management, such as initiation, shifting and working memory that may contribute to daily functioning with CD [41]. Understanding HRQOL along with deeper understanding of children’s and adolescents’ daily activities and participation characteristics and specific cognitive skills, may serve as important measures in follow-up.

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Chapter 2 **– Wheat amylase-trypsin inhibitors (ATI) as potent triggers of innate intestinal immunity in patients with celiac disease and non-celiac gluten sensitivity (NCGS)**

1.1. BACKGROUND

NCGS is a non-allergic and non-autoimmune condition, characterized by intestinal and/or extraintestinal symptoms caused by gluten containing food ingestion (wheat, barley, rye), diagnosed after exclusion of both CD and WA. It is a recently described disorder, with an estimated prevalence of around 2% in the general population [1]. Over recent decades, the prevalence of gluten related disorders has increased in developed countries and this finding points to the role of one or more possible environmental triggers other than gluten [2-5]. The interplay between genetic and environmental factors regulating the balance between tolerance and immune response to gluten is still poorly understood. It has been recently hypothesized that the type of gluten contained in modern grains and its immunogenic properties significantly differ from the ancient varieties and this may have influenced the increased prevalence of gluten related disorders [6-8]. Wheat is ubiquitous, one of the most used grain in the world, present in the human diet particularly after the spread of agriculture accompanied by the domestication of wild plant. The domestication of wheat started from the first domesticated hulled grains as eikorn and emmer about 10,000 years ago from the Fertile Crescent. Tetraploid wheats, *Triticum turgidum* L. (AABB genome), were domesticate in the Fertile Crescent with the development of emmer (*T. turgidum* ssp. *dicoccum*) alongside with eikorn, thew diploid wheat (*Triticum monococcum*; AA genome) and barley. They offer an interesting model to study the effects of selection associated to domestication. Emmer has spread following human migrations throughout Europe and Asia and became the most important crop in Fertile Crescent until the early Bronze Age. Free-threshing tetraploid wheats subsequently originated from emmer. This event was followed by the selection of durum wheat (*T. turgidum* ssp. *Turgidum convar. durum*), as crop specialized for the production of pasta, cous-cous, traditional/typical bread and bulgur, and its spread in the Mediterranean region. It is

reasonable to consider the evolution of tetraploid wheats as consisting of at least two steps: (i) the primary domestication, with the passage from wild-emmer (*T. turgidum* ssp. *dicoccoides*) to emmer, and (ii) the secondary domestication, from emmer to durum wheat. In parallel, after an interspecies natural hybridization between a tetraploid domesticated wheat and the wild *Aegilops tauschii* followed by a spontaneous poliploidization, an hexaploid wheat (AABBDD genome) was developed with the formation of spelt (*Triticum spelta*), and bread wheat (*Triticum aestivum*). The introduction of the D-genome improved the yield and bread-making properties. Further breeding over past centuries led to a change in the gluten content quality and composition, enhanced crop yield, and nutritional properties important for the application of tetraploid durum and hexaploid bread wheat in food production. Particularly these modifications have increased the gluten content and led to a "strong" gluten, in terms of visco-elasticity, properties required to process semolina into a suitable final product with an optimal cooking performance. Durum and bread wheat varieties are widely used in current time to produce pasta and bread respectively. There is preliminary evidences suggesting that early domesticated wheats (diploid or tetraploid) may be safer and less immunogenic compared to presently used hexaploid and durum wheat [6-8]. However, such analyses were conducted in a very limited sample of wheat genotypes, making difficult to clarify if specific wheat taxa may represent a source of genes to be used to improve modern varieties. The aim of the study was: (i) to compare the immunological properties of different taxa that represent the whole evolution history of wheat varieties in dendritic cells collected from CD patients at diagnosis, treated CD patients, treated NCGS patients and healthy controls; (ii) to identify wheat varieties characterized by absent or low toxicity of gluten, that could be tested in clinical studies on patient with NCGS; (iii) to select wheat varieties that could find wider use in human nutrition with the aim of preventing NCGS and other gluten related disorders; (iv) to select wheat varieties that could be cultivated in the Marche Region in replacement of varieties that are more toxic for genetically predisposed individuals.

1.2. METHODS

A total of 19 different genotypic varieties of wheat were selected, which belong to 6 taxonomic families used in different periods and which therefore represent the whole evolution history of wheat. In particular, 5 genotypic varieties of wheat were selected for each of the 3 main historical stages pre-domestication, first domestication, second domestication: (i) *Triticum turgidum* ssp. *dicoccoides* (or wild spelled) (varieties: ttd1, ttd5, ttd6, ttd9, ttd10), (ii) *T. turgidum* ssp. *dicoccum* (or domesticated spelled) (varieties: f6, f7, f10, f3, f4) and (iii) *T. turgidum* ssp. *durum* (or durum wheat) (varieties: fd2, fd6, fd8, fd10, fd12). Three different replicas were selected for each variety (i.e. varieties grown in different locations: Potenza, Ancona, Julich-Germany), to verify the phenotypic differences attributable to different environmental factors. Finally, two varieties of diploid wheat were selected, *T. monococcum* (or small spelled) variety ID3 and Hammurabi, which is the first wheat cultivated in history, and two varieties of hexaploid wheat (or modern wheat): *T. aestivum* ssp. *aestivum* or "soft wheat" (Botticelli variety) and *T. aestivum* ssp. *Spelta* (ALT GOLD variety). In total, 49 varieties of wheat were selected. The grain samples studied are shown in Table 1.

Table 1. List of wheat varieties.

Code	Replicate	Species	Notes	AN Code
AN_BAZ382	AN	*Triticum turgidum ssp. dicoccoides*		**ttd1**
PZ_BAZ382	PZ	*Triticum turgidum ssp. dicoccoides*		**ttd1**
JU_BAZ382	JU	*Triticum turgidum ssp. dicoccoides*		**ttd1**
AN_PI352324	AN	*Triticum turgidum ssp. dicoccoides*	Lebanon	**ttd5**
PZ_PI352324	PZ	*Triticum turgidum ssp. dicoccoides*	Lebanon	**ttd5**
JU_PI352324	JU	*Triticum turgidum ssp. dicoccoides*	Lebanon	**ttd5**
AN_PI355459	AN	*Triticum turgidum ssp. dicoccoides*	Armenia	**ttd6**
PZ_PI355459	PZ	*Triticum turgidum ssp. dicoccoides*	Armenia	**ttd6**
JU_PI355459	JU	*Triticum turgidum ssp. dicoccoides*	Armenia	**ttd6**
AN_PI470944	AN	*Triticum turgidum ssp. dicoccoides*	Syria	**ttd9**
PZ_PI470944	PZ	*Triticum turgidum ssp. dicoccoides*	Syria	**ttd9**

JU_PI470944	JU	*Triticum turgidum ssp. dicoccoides*	Syria	**ttd9**
AN_PI481539	AN	*Triticum turgidum ssp. dicoccoides*	Israel	**ttd10**
PZ_PI481539	PZ	*Triticum turgidum ssp. dicoccoides*	Israel	**ttd10**
JU_PI481539	JU	*Triticum turgidum ssp. dicoccoides*	Israel	**ttd10**
AN_FARVENTO	AN	*Triticum turgidum ssp. dicoccum*	Italy	**f6**
PZ_FARVENTO	PZ	*Triticum turgidum ssp. dicoccum*	Italy	**f6**
JU_FARVENTO	JU	*Triticum turgidum ssp. dicoccum*	Italy	**f6**
AN_LUCANICA	AN	*Triticum turgidum ssp. dicoccum*	Italy	**f7**
PZ_LUCANICA	PZ	*Triticum turgidum ssp. dicoccum*	Italy	**f7**
JU_LUCANICA	JU	*Triticum turgidum ssp. dicoccum*	Italy	**f7**
AN_MG5350	AN	*Triticum turgidum ssp. dicoccum*	Ethiopia	**f10**
PZ_MG5350	PZ	*Triticum turgidum ssp. dicoccum*	Ethiopia	**f10**
JU_MG5350	JU	*Triticum turgidum ssp. dicoccum*	Ethiopia	**f10**
AN_PI470739	AN	*Triticum turgidum ssp. dicoccum*	Turkey	**f3**
PZ_PI470739	PZ	*Triticum turgidum ssp. dicoccum*	Turkey	**f3**
JU_PI470739	JU	*Triticum turgidum ssp. dicoccum*	Turkey	**f3**
AN_PI74106	AN	*Triticum turgidum ssp. dicoccum*	Iran	**f4**
PZ_PI74106	PZ	*Triticum turgidum ssp. dicoccum*	Iran	**f4**
JU_PI74106	JU	*Triticum turgidum ssp. dicoccum*	Iran	**f4**
AN_CAPEITI_8	AN	*Triticum turgidum ssp. durum*	Italy	**fd2**
PZ_CAPEITI_8	PZ	*Triticum turgidum ssp. durum*	Italy	**fd2**
JU_CAPEITI_8	JU	*Triticum turgidum ssp. durum*	Italy	**fd2**
AN_NEODUR	AN	*Triticum turgidum ssp. durum*	France	**fd6**
PZ_NEODUR	PZ	*Triticum turgidum ssp. durum*	France	**fd6**
JU_NEODUR	JU	*Triticum turgidum ssp. durum*	France	**fd6**
AN_PEDROSO	AN	*Triticum turgidum ssp. durum*	Spain	**fd8**
PZ_PEDROSO	PZ	*Triticum turgidum ssp. durum*	Spain	**fd8**
JU_PEDROSO	JU	*Triticum turgidum ssp. durum*	Spain	**fd8**
AN_SIMETO	AN	*Triticum turgidum ssp. durum*	Italy	**fd10**
PZ_SIMETO	PZ	*Triticum turgidum ssp. durum*	Italy	**fd10**
JU_SIMETO	JU	*Triticum turgidum ssp. durum*	Italy	**fd10**
AN_TRINAKRIA	AN	*Triticum turgidum ssp. durum*	Italy	**fd12**
PZ_TRINAKRIA	PZ	*Triticum turgidum ssp. durum*	Italy	**fd12**
JU_TRINAKRIA	JU	*Triticum turgidum ssp. durum*	Italy	**fd12**
GEPPETTO		*Triticum aestivum ssp. aestivum*	Italy	
ID3		*Triticum monococcum ssp. monococcum L.),*		

ALT GOLD ROTKORN		*Triticum aestivum ssp. Spelta*		

The final dataset included 15 cultivars of 3 genotypes (*Triticum turgidum* ssp. *durum, Triticum turgidum* ssp. *dicoccoides, Triticum turgidum* ssp. *dicoccum*) collected from three distinct production areas (Potenza - PZ, Ancona - AN, Julich – JU Germania) and one cultivar of *Triticum monococcum* (Einkorn). The protein fraction of interest was extracted from the flours according to the procedure reported by Zevallos et al. [9] Briefly, the flours were obtained by grinding individual grain samples in granite mortars in the presence of liquid nitrogen, and 10 grams of each flour were deprived of the fat component by extraction with a mixture of CH3OH/C2H5OC2H5 (flour weight/extraction solution volume ratio: 1/10) for 2 hours at room temperature. The suspension was then centrifuged at 2500 x g for 20 minutes, the pellet collected, dried overnight, and subjected to three successive extraction cycles at 4 ° C in NH4HCO3 50 mM pH 7.8 (flour weight/extraction solution volume ratio: 1/5), to maximize the yield of the procedure. The suspensions were centrifuged at 2500 x g for 20 minutes, and the corresponding supernatants mixed. These solutions were subjected to fractional precipitation in (NH4) 2SO4 at 4 °C, the fraction precipitated between 1.8 M and 4 M (NH4) 2SO4 was collected, extensively dialyzed in NH4HCO3 10 mM pH 7.8 (MWCO: 3.5 kDa), aliquoted and stored at -20 °C until use. The protein concentration of each solution was determined by the Bradford method. The solutions showed protein concentrations ranging from 1.8-11 mg per gram of flour. The chromatographic separation of protein mixtures was carried out through an adaptation to FPLC chromatography of the method proposed by Zevallos et al. [9]. The protein solutions obtained from each wheat sample were filtered through cellulose esters membrane filters (0.22 µm) and separated by molecular exclusion chromatography on an AKTA Basic FPLC system equipped with Superdex 75 HR 10/300 GL column (GE Healthcare Lifesciences). Each fraction has been further characterized by shotgun protein identification using electrospray ionization-ion trap mass spectrometry, in positive ion mode. The individual main fractions were lyophilized and stored at -20 °C until use.

A detailed protocol was developed by optimizing the techniques for cell culture and the differentiation of dendritic cells from mononuclear cells isolated from the peripheral blood of healthy donors, and the panel of antibodies and fluorochromes to be used for the surface marking of cells was established. Furthermore, several experiments were carried out testing various concentrations of LPS and ATI, to find the optimal conditions for lymphocytes and dendritic cells stimulation. Finally, the best panel to analyze the cytokines produced by dendritic cells in culture, by means of Multiplex ELISA was established.

The peripheral blood mononuclear cells were separated by Ficoll and cultured in RPMI medium supplemented with 10% heat decomplemented FBS (fetal bovine serum) in the presence of IL-4 and GM-CSF cytokines, to stimulate the adhesion of monocytes and their subsequent differentiation into dendritic cells. After 2 weeks of incubation (37 °C, 5% CO2) the differentiated dendritic cells were incubated with LPS or ATI for 16 hours, in the presence of IFN-γ. These cells were then scraped from the plate surface and analyzed on a flow cytometer (BD FacsCanto II), marking them for the detection of the following surface molecules: CD80, CD83, CD86, CD25. The supernatants of all cell cultures were stored at -20 °C until further analysis by a Multiplex ELISA to study the following cytokines: IL10, IL17, IL-18, IL-1β. IL-4, IL-6, IL-8, MCP-1, RANTES, TNF-α, TRAIL. The following ATI were tested: FD8, FD12, FD10, FD2, FD6, F7, F3, F6, F4, F10, TTD1, TTD5, TTD6, TTD10, TTD9. Each ATI was tested on 3 or more different donors (LPS concentration 40 ng/ml; ATI concentration 150 µg/ml). THP-1 cells transfected with the TLR4-CD14-MD2 complex (InvivoGen) were cultured in RPMI or DMEM (Cellgro) supplemented with 100 IU/ml penicillin/100 µg/ml streptomycin and 10% FBS at 37 °C in a 5% CO2 atmosphere. All cell lines were tested for mycoplasma and no contamination was detected. ATI extracts reconstituted in PBS (phosphate-buffered saline) solution were added to the ATI-reactive (TLR4-bearing) cell cultures (THP-1, U937). It was also demonstrated that the ammonium bicarbonate in which the ATI were solubilized did not negatively affect the treatment and did not increase inflammatory cytokines levels.

1.3. RESULTS

Different amounts of ATI were found based on different wheat genotypes and different production areas. The deconvolution analysis of the chromatograms (Marquadt-Levemberg data fitting using a model based on multi-Gaussians on Python 3.5.2) revealed the presence of 10 chromatographic distinguishable fractions with molecular weights in the range 1-350 kDa shared by all samples, as shown by the low standard deviation values associated with the retention volumes of each peak. While presenting a qualitatively similar protein content, the different varieties had peculiar chromatographic profiles that differed in the relative abundance of the individual components. The results of the chromatographic analysis are shown in Table 2.

Table 2. Individual ATI protein content.

Name	ID	PROV	GENE	CONC (mg/g flour)
PI352324	TTD	PZ	TTDC	4,76
NEODUR	FD	JU	TTDU	6,68
PI352324	TTD	JU	TTDC	4,89
CAPEITI	FD	JU	TTDU	5,84
SIMETO	FD	PZ	TTDU	2,94
PI355459	TTD	JU	TTDC	5,15
MG5350	F	AN	TTDI	4,36
PEDROSO	FD	AN	TTDU	5,07
LUCANICA_CORRETTO	F	PZ	TTDI	2,48
PEDROSO	FD	PZ	TTDU	4,13
PI470944	TTD	PZ	TTDC	5,38
CAPEITI8	FD	AN	TTDU	3,39
NEODUR	FD	PZ	TTDU	3,18
PI74106	F	PZ	TTDI	2,63
PI470739	F	JU	TTDI	2,00
BAZ382	TTD	JU	TTDC	4,21
PI481539	TTD	JU	TTDC	5,29
PEDROSO	FD	JU	TTDU	8,00
PI355459	TTD	AN	TTDC	4,28
PI481539	TTD	PZ	TTDC	3,14
FARVENTO_CONC	F	PZ	TTDI	1,59
BAZ382	TTD	AN	TTDC	5,29
TRINAKRIA	FD	JU	TTDU	6,07

Triticum monococcum ssp. Monococcum L.				4,68
SIMETO	FD	JU	TTDU	6,93
PI74106	F	JU	TTDI	2,03
Triticum aestivum ssp. Spelta				5,29
PI74106	F	AN	TTDI	2,92
Triticum aestivum ssp. aestivum				3,91
NEODUR	FD	AN	TTDU	4,00
LUCANICA	F	AN	TTDI	3,75
FARVENTO	F	JU	TTDI	3,97
FARVENTO	F	AN	TTDI	3,53
PI355459	TTD	PZ	TTDC	2,94
TRINAKRIA	FD	AN	TTDU	5,41
CAPEITI8002	FD	PZ	TTDU	5,96
BAZ382	TTD	PZ	TTDC	2,18
PI352324	TTD	AN	TTDC	5,45
LUCANICA	F	JU	TTDI	2,45
MG5350	F	JU	TTDI	5,05
MG5350	F	PZ	TTDI	3,06
PI470739	F	AN	TTDI	3,93
PI470944	TTD	JU	TTDC	4,06
SIMETO	FD	AN	TTDU	3,38
PI470944	TTD	AN	TTDC	8,23
PI470739	F	PZ	TTDI	1,86
PI481539	TTD	AN	TTDC	4,23
TRINAKRIA	FD	PZ	TTDU	1,48

All the samples present the same chromatographic pattern (Figure 1), while different phenotypes (Figure 2) and genotypes (Figure 3) present statistically significant differences between the concentrations of each fraction.

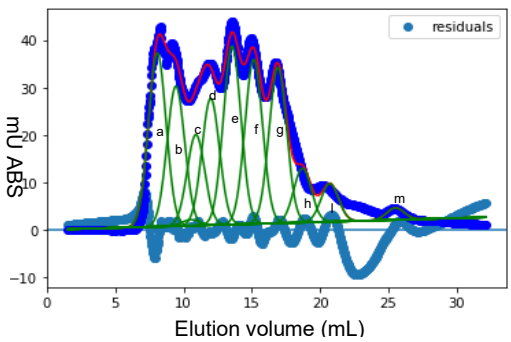

Figure 1. Chromatographic profile of BAZ382-PZ using a multi-Gaussian model.

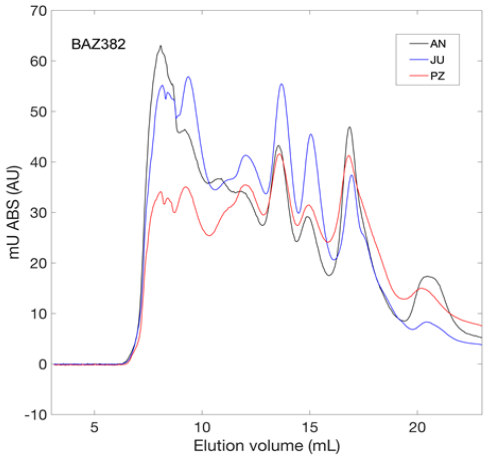

Figure 2. Chromatographic profile of BAZ382 from three different geographical regions.

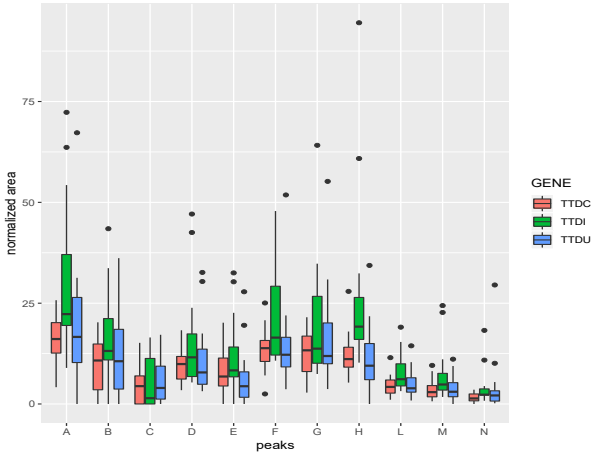

Figure 3. Abundances of each peak comparing three different genotypes.

All wheat species from Potenza were able to activate dendritic cells causing both an increase in the expression of surface markers, especially CD83 and CD86, and a statistically significant increase in the production of all the cytokines studied.

All wheat species from Ancona were able to activate dendritic cells causing an increase in the expression of the surface markers CD80, CD83 and CD86. The production of IL6, IL8 and

MCP1 cytokines was significantly increased for all ATI studied, while the production of IL4, IL10, IL18, RANTES and TRAIL cytokines was significantly lower than controls.

All wheat species from Germany were able to activate dendritic cells causing both an increase in the expression of surface markers, especially CD25, CD80 and CD83, and a statistically significant increase in the production of all cytokines studied. Experiments on THP-1 cells revealed that F7-Lucanica, F4-PI74106, F6- FARVENTO varieties from Potenza were able to increase the release of pro-inflammatory cytokines (IL1B, TNFa, IL6 , IL8, IL4 and IL10) while others, particularly TTD6 (species of wild spelled PI355459), showed absent/significantly lower inflammatory activity. According to the production area, the experiments on THP1 cells and dendritic cells showed that the wheat varieties cultivated in Potenza induce a greater production of pro-inflammatory cytokines, compared to the varieties cultivated in Germany and especially to those cultivated in Ancona. According to the genotypes, the results on cell lines and dendritic cells showed that ATI with lower biological activity belong to the family of Triticum turgidum ssp. Dicoccoides, in particular the TTD6 variety, while the greatest biological activity was found in ATI belonging to the family of Triticum aestivum ssp aestivum and ssp spelta and subsequently of *Triticum turgidum* ssp. *durum* (particularly fd8 variety), and of *Triticum turgidum* ssp. *dicoccum* (particularly F7 and F4 varieties). About *Triticum monococcum*, the results are controversial. Experiments on dendritic cells show that both tested varieties, Hamurrabi and ID3, were unable to induce either the expression of surface markers of dendritic cells, or the production of pro-inflammatory cytokines. However, the experiments on THP1 cells following incubation with the two varieties of Einkorn showed a significant increase in cytokines levels (IL6, IL1b, TNFa, MCP1, IL4, IL18, TRAIL) compared to both the LPS control and the other wheat varieties tested. Finally, a significant correlation was found between the amount of ATI observed with chromatographic analysis and their biological activity demonstrated by in vitro studies, so that the varieties with lower amounts of ATI induce a lower production of pro-inflammatory cytokines. The study is currently ongoing.

1.4. DISCUSSION

Preliminary results demonstrated that there are different concentrations of ATI in different wheat genotypes or in the same genotype cultivated in different areas, and that there is a significant correlation with their biological activity, suggesting that both wheat genotype and phenotype can influence the immune response [10]. In addition, some varieties with lower biological activity were identified and they will be used in a clinical trial involving subjects with NCGS to verify the in vivo tolerance.

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Chapter 3 **– Quantification of accidental gluten contamination in the diet of children with treated celiac disease *(Nutrients 2021)***

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1.1. BACKGROUND

Celiac disease (CD) is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in genetically predisposed individuals. A strict and permanent gluten-free diet (GFD) is the only effective treatment for CD. The GFD determines disappearance of celiac-related symptoms and serum autoantibodies, recovery of intestinal mucosa, and prevention of long-term complications [1]. Both children and adults with CD are highly sensitive to the toxic effects of gluten exposure. It has been shown that the protracted ingestion of gluten traces (>10 mg/day) is sufficient to cause significant damage in the architecture of the small intestinal mucosa in patients on treatment for CD. Based on this threshold, a maximum tolerable amount of gluten of <20 parts per million (ppm) in gluten-free food has been calculated [2], a limit that has been endorsed by the major international regulatory agencies, e.g., the Codex Alimentarius, the US Food and Drug Administration (FDA), and the European Food Safety Authority (EFSA) [3-5]. Deviation from the GFD is unfortunately easy, due to both voluntary and inadvertent dietary transgressions. Gluten is indeed a pervasive ingredient that may be used as a protein filler in many commercial food (e.g., sausages, soups, soy sauces, etc.) or may contaminate originally gluten-free products (e.g., oats and legumes) during the production chain.

In recent years, several studies from different countries investigated the level of gluten contamination in foodstuff [6-11], but only few data are available on the daily intake of contaminating gluten in treated CD patients.

The aim of the present study was to directly measure the level of contaminating gluten in the diet of CD children followed at our Celiac Center.

1.2. METHODS

Study Group

From April 2019 to December 2019, CD children (2–18 years old) on GFD for ≥6 months attending medical follow-up visits at our Celiac Center were offered to participate in the study. The initial diagnosis of CD was performed according to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines [12]. Patients who had comorbidities requiring additional dietary restrictions, particularly Type 1 Diabetes, inflammatory bowel diseases, or food allergies, were excluded from participation. Written informed consent was obtained from parents of participating children, and additional written assent was obtained from age-eligible children. The study was conducted in accordance with the principles of the Helsinki Declaration as revised in Fortaleza 2013 and was approved by the ethical committee of the Polytechnic University of Marche (ID # 124827).

Study Design

Participants were encouraged to maintain their usual eating pattern during the diet sampling period. The weekday of diet sampling was randomly assigned at enrollment. Patients and their caregivers were invited to provide a representative portion (about 10 g) of all meals consumed during the 24-h period. They were requested to weigh all ingested food using a kitchen scale and to report the amount, the composition, and other details (including ingredients, food type, place, and time of sampling) of each meal/snack on the 24-h food diary. Each subject was provided with sterile plastic bags and cups to collect food portions. Samples from breakfast,

lunch, snacks, and dinner were included. Naturally gluten-free, unprocessed food (e.g., water, milk, fruits, and raw vegetables) were not collected. Samples were given a unique laboratory code, and were stored at −20 °C until analysis.

Determination of Gluten Content in Food Samples by R5 Ridascreen ELISA

All food samples were processed for gluten content determination by the Ridascreen Gliadin sandwich R5 enzyme-linked immunosorbent assay (ELISA) R-7001 (R-Biopharm, Darmstadt, Germany) at our Celiac Disease Research Laboratory, Polytechnic University of Marche, Ancona. During each run of ELISA, the manufacturer's guidelines were strictly followed. The Ridascreen R5 ELISA was performed as previously described [6].

Gluten Quantification

The gluten content of analyzed food samples was expressed as ppm. The lower limit of quantification was 5 ppm of gluten. All products with a gluten level higher than 20 ppm were re-extracted and analyzed for a second time.

Finally, we estimated the 24-h amount of gluten consumed by participating children using the following formula to convert ppm of gluten into mg of gluten/day for all the meals with measurable gluten contamination: mg/day gluten = ppm gluten in the food portion × food sample weight (g)/1000.

Determination of Serum IgA Anti-Tissue Transglutaminase Antibody

IgA anti-tissue transglutaminase (anti-tTG) antibody assay was performed in all participating children in our Laboratory by fluorescence enzyme immunoassay ≤30 days prior to the start of the study (normal values <7 U/mL) as part of routine follow-up visits.

Statistical Analysis

Data are presented as medians (range) or percentages, as appropriate. The sample size was estimated on the basis of the expected prevalence of gluten exposure. GraphPad Prism

software (version 7, GraphPad Software, La Jolla, CA, USA), and Microsoft EXCEL (v.2010; Microsoft Corp Redmond, Washington, DC, USA) were used for the analysis.

1.3. RESULTS

Of the 94 eligible pediatric CD patients, 25 children were excluded because of concomitant diseases (n = 5) or declined participation (n = 19) or incomplete collection of samples (n = 1). Sixty-nine children completed the protocol. Demographic and clinical data of these patients are shown in Table 1.

Table 1. Demographic and clinical data of the study-group.

Variable	CD Children (*n* = 69)
Gender	27 M (39%)/42 F (61%)
Median age (years)	9 (range: 2–18)
Median disease duration (years)	2 (range: 0.5–9)
■ <1 year	27/69 (39%)
■ 1–2 years	13/69 (19%)
■ >2 years	29/69 (42%)
Positive IgA anti-tTG [1]	27/69 (39%)
■ <1 year after diagnosis	19/27 (70%)
■ 1–2 years after diagnosis	4/27 (15%)
■ >2 years after diagnosis	4/27 (15%)

[1] IgA anti-tTG, IgA anti-tissue transglutaminase.

No intentional gluten exposure was reported during the 24-h period of diet sampling by these patients and their caregivers.

Each patient provided 7 food samples on average. A total of 448 food samples were provided from these 69 subjects. Samples belonged to the following food categories: “Pasta and bakery products” (46%) including pasta, lasagna, rice, pizza, wraps, crackers, breadsticks, sandwiches, and stuffed focaccia; “Sweet snacks” (26%) including biscuits, cakes, nougats, ice-creams, muesli, waffles, cornflakes, and chocolate tarts; “Meat/fish-based products” (20%) including cooked meat/fish, cold cuts, eggs, cheeses, yogurts, and mayonnaise; “Vegetable-based products” (8%) including cooked vegetables, processed fruits, legumes, and vegetable soups. Meals including foods from more than one food group were assigned to a specific

category on the basis of the most represented ingredient. In total, 299 samples were collected at home, 76 at relatives' home, 61 at school, and 12 at restaurants. Of them, 316 were collected on weekdays and 132 during the weekend. The level of gluten contamination in the analyzed samples according to the different settings of consumption is shown in Table 2.

Table 2. Gluten contamination level according to food samples features.

	Gluten Contamination		
	<5 ppm *n* **= 436 (97%)**	**5–20 ppm** *n* **= 11 (2.8%)**	**>20 ppm** *n* **= 1 (0.2%)**
Place of sampling			
■ Home	336 (97%)	9 (3%)	0 (0%)
■ Relatives	73 (96%)	2 (3%)	1 (1%)
■ School	15 (100%)	0 (0%)	0 (0%)
■ Restaurant	12 (100%)	0 (0%)	0 (0%)
Time of sampling			
■ Weekdays	308 (97%)	7 (2%)	1 (0.3%)
■ Weekend	128 (97%)	4 (3%)	0 (0%)

"Home" included all the meals prepared and consumed at home. "Relatives" included meals prepared and consumed at grandparents' and uncles' home. "School" included meals prepared at the school canteen. "Restaurant" included meals consumed in restaurants, pizzerias, sandwich shops, and ice-cream parlors.

Overall, 12/448 (2.7%) food samples showed detectable gluten contamination; of these, 11 contained gluten within tolerable limits (5–20 ppm) and only one contained >20 ppm of gluten (Figure 1).

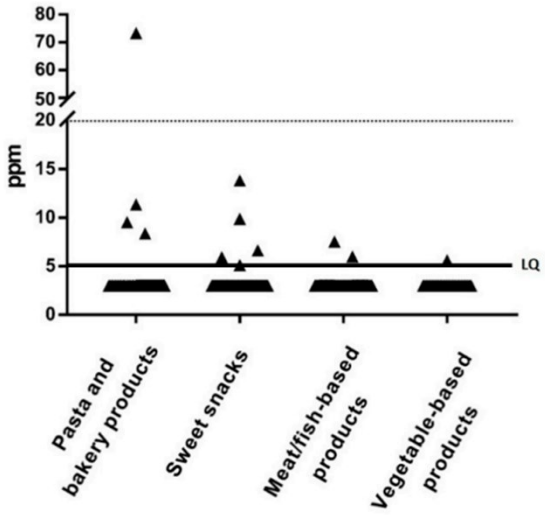

Figure 1. Gluten content in the 448 analyzed samples according to the food group. LQ, limit of quantification (solid line).

The median concentration of gluten in positive samples was 8 ppm (range: 5 to 74 ppm). The 12 contaminated food samples were from 5 of the 69 enrolled patients (7%; 1 male and 4 females): 2 patients had only 1 contaminated meal (total level of gluten contamination/day was 1.86 mg and 0.18 mg, respectively), 2 patients had 2 contaminated meals (total level of gluten contamination/day was 0.39 mg and 0.58 mg, respectively), and 1 had 6 contaminated meals (total level of gluten contamination/day was 3.61 mg). Two of these 5 patients showed IgA anti-tTG antibodies positivity: one had 7-fold and the other 1-fold higher levels than the upper normal value (cutoff: 7 U/mL). No significant difference was found in the percentage of anti-tTG antibody positivity according to the presence of gluten contamination in the diet ($p = 0.664$). Three of the 12 contaminated items were from children aged 2–5 years while 9 were from children aged 6–10 years. No contaminated items were found in subjects aged 11–18 years. The only food sample contaminated with more than 20 ppm of gluten was from a 2-year-old female patient, and was prepared and consumed at the grandmother's home (total level of gluten contamination/day was 1.86 mg). In the 5 children ingesting contaminated foodstuff, the daily gluten intake was always well below the safety threshold of 10 mg/day (3.61, 1.86, 0.58, 0.39, and 0.18 mg/day, respectively).

1.4. DISCUSSION

To our knowledge, this is the first study to quantify the amount of inadvertent gluten exposure in treated CD patients. In our sample of 69 Italian CD children on GFD regularly followed-up, we found that gluten contamination of the GFD was extremely rare (only one food sample showing >20 ppm of gluten out of 448 analyzed) and almost negligible on a quantitative basis. Only 5 out of 69 celiac children (7%) ingested gluten traces during the 24-h test-period, and the total amount of gluten contamination (0.2–4 mg/day) was always well below the tolerable threshold (10 mg/day) in these cases. A strict GFD is extremely difficult to maintain since gluten may contaminate many different commercial food items. The only method to quantify traces of gluten in the GFD is the analysis of ingested food by a reference analytical method

associated with quantification of food portions consumed during a given period of time, e.g., 24 h, as performed in the present study. The ELISA R5 used here is currently classified as a Codex type I method for gluten determination in foods and, therefore, represents the most widely used assay [13]. The R5 antibody accurately detects prolamins of wheat (gliadins), rye (secalins), and barley (hordeins), in both raw flours and processed food products [14]. Limitations of this method are the potential interference of different food matrices with antibody binding, and the poor reliability in measuring hydrolyzed gluten in beer, a dietary component that was not consumed by our pediatric patients. R5 ELISA is the only certified method that has been endorsed by several international agencies including the Codex Alimentarius, US FDA, and the European EFSA [3-5].

Our favorable results may be explained by several factors: (a) inclusion of highly compliant patients who are regularly seen at the Celiac Clinic; (b) generalized conformity of GF products marketed in Italy with the international regulations for labeled gluten-free food [6]; (c) high level of awareness of the requirement of the GFD by the general population in Italy, particularly due to the national Celiac Protection law (n.123/2005) and the pro-active role of the Italian Celiac Association that strongly helps families in managing the daily needs of the GFD, for example, by a capillary surveillance of restaurants and pizzerias.

A higher frequency of food samples contaminated with >20 ppm of gluten (3%) was recently reported by Silvester et al. in Canadian adults with CD, however the overall daily intake of contaminating gluten was not reported in that study [15]. Higher rates of poor adherence to the GFD have been reported in studies based on indirect evaluation of contaminating gluten. Stefanolo et al. [16] investigated the patterns of gluten exposure during a 4-week period, as assessed by GIP excretion in urine and stool. These authors reported a high rate of inadvertent gluten exposure in CD patients, with 89% of patients excreting GIP in either stool and/or urine at least once during the four-week period. It should however be noted that the relationship between urinary GIP positivity and the amount of ingested gluten is still unclear. Furthermore, in that study, samples were collected only during the weekend, when people frequently dine out. Syage et al. [17] estimated that the mean daily gluten consumption of

children following a GFD was 387 mg/day. These estimates were based on two assumptions, the conversion factor from GIP to gluten ingestion and the equation describing the relationship between the dose of ingested gluten and the morphometric change of the small intestinal mucosa, that have not been verified so far.

A significant proportion of our patients showed positivity of IgA class anti-tTG antibodies determination, a finding that might suggest persisting active disease caused by ongoing gluten ingestion. However, this result does not conflict with the excellent adherence to the GFD that we observed in our study-group. Previous studies in treated celiacs have consistently shown that the correlation between CD serology results and dietary evaluation of compliance to the GFD is poor [18]. Even more importantly, most of our patients (85%) with anti-tTG positivity were investigated during the first two years of GFD treatment. It is well established that normalization of IgA anti-tTG levels after starting the GFD may take longer than two years in a significant proportion of cases [19].

Strengths and Limitations

The strengths of our study are the direct determination of contaminating gluten, the prospective registration of ingested food, the large sample of analyzed food, and the use of a reference method to quantify gluten in different food matrices. The limitations are the selection bias introduced by investigating children and families highly compliant with the CD follow-up schedule, and the possible modification of the usual dietary behavior in response to the awareness of being under investigation (so called Hawthorne effect). This is an unavoidable bias in dietary prospective studies like ours.

1.5. CONCLUSIONS

In a group of Italian children strictly following the CD follow-up program, the daily unintended exposure to gluten was very low, and did not lead to exceed the tolerable threshold of 10 mg/day of gluten intake in the GFD.

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Chapter 4 **– Determination of urinary gluten immunogenic peptides to assess adherence to the gluten-free diet: a randomized, double-blind, controlled study *(Clin Transl Gastroenterol. 2021)***

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1.1. BACKGROUND

Celiac disease (CD) is a systemic autoimmune disorder triggered by the ingestion of gluten in genetically predisposed individuals [1]. It is one of the most frequent lifelong diseases, affecting approximately 1%–2% of the general population worldwide [2]. A gluten-free diet (GFD), the only effective treatment of CD, determines clinical, serological, and histological remission and prevents long-term CD complications [3]. However, a strict GFD is extremely difficult to maintain. Gluten is indeed a pervasive ingredient that may be used as a protein filler in a huge number of commercial foods (e.g., sausages, soups, soy sauces, and hamburgers) or may contaminate originally gluten-free products in the production chain [4]. Unfortunately, even traces of gluten in the diet (≥10 mg/d) are sufficient to cause damage to the celiac small intestinal mucosa when ingested repeatedly [5].

Hence, it is very important to monitor GFD adherence of patients with CD. Dietary interview, clinical symptoms monitoring, CD serology, and small intestinal histology are significant choices; however, they provide only limited and indirect evidence of GFD adherence [6–8]. Moreover, these tools are inadequately sensitive to detect the accidental exposure to traces of dietary gluten. Novel qualitative and quantitative immunochromatographic tests have been

developed to directly detect recent dietary exposure to gluten by determining the excretion of gluten immunogenic peptides (GIP) in stools or urine [9–11]. A growing interest has recently focused on the role of stool/urinary GIP determination in the follow-up of treated patients with CD, and this noninvasive and easy to perform test seems to be the most promising and reliable marker of dietary gluten transgressions [12–22]. Inadequate information is available about the relationship between the amount of ingested gluten and the quantity of GIP excreted in urine or stool particularly at a low level of gluten ingestion (as is usually the case in treated patients with CD). The aim of this study was to assess the diagnostic performance of urinary GIP determination and the dose-response relationship between the amount of ingested gluten and the quantity of GIP recovered in urine, in a group of healthy and qualified volunteers adhering to a GFD and undergoing repeated dietary challenges with increasing amounts of gluten.

1.2. METHODS

This study was a randomized, double-blind, controlled study aimed to investigate the relationship between the increasing amount of ingested gluten and the quantity of GIP in urine.

Participants

This study was conducted on a group of healthy young medical doctors who were all pediatric residents in the Division of Pediatrics at the DISCO Department of the Polytechnic University of Marche, Ancona, Italy. Written informed consent was obtained from each participant. The exclusion criteria were any chronic or acute disease, pregnancy or lactation, chronic intake of medications or supplements, or refusal/withdrawal of written informed consent. Before the study, serum immunoglobulin A (IgA) class anti-transglutaminase antibody was determined in all participants to exclude active CD.

Study design

Each participant underwent a random sequence of single-dose gluten challenges, collection of all urine excreted during the following 24 hours, and quantification of urinary GIP. Recent

data showed that urinary GIP are undetectable after 16–34 hours from the complete removal of gluten from the diet [11]. Therefore, to guarantee the complete absence of urinary GIP at baseline, a strict GFD was started 3 days (72 hours) before each gluten challenge and continued for 24 hours after the gluten challenge. A urine sample was collected at T0 (first morning urine after 3 days of GFD and immediately before the gluten challenge). After the gluten challenge, all urine excreted during the next 24 hours was collected into 2 different sterile containers (1 for the first 9 hours [T0-T9 collection] and the other for the following 13 hours [T10-T24 collection]), and the total volumes were measured. Sample timing (T9 and T24) was based on previous data suggesting that GIP is detected in urine between 3 and 9 hours from gluten reintroduction [11].

This study consisted of 2 parts (A and B), each characterized by a different approach to the GFD and by different gluten doses administered with the challenge. During study A, all participants were instructed to follow 6 bouts of a standard GFD (see Dietary Interventions section). The participants were randomized to a sequence of 6 gluten challenges (0, 10, 50, 100, 500, and 1,000 mg of gluten) (Figure1).

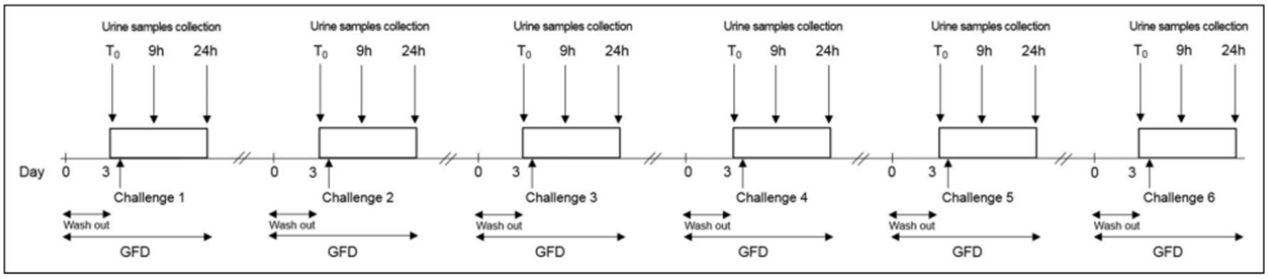

Figure 1. Flow chart of study A. GFD, gluten-free diet; T_0, baseline.

The gluten was administered in capsules prepared by our hospital pharmacy. Each capsule contained a weighed amount of raw gluten.

Study B was deemed necessary after analyzing the results of study A (see Results section). In study B, a subgroup of randomly chosen participants underwent 2 further gluten challenges in a random sequence with either 5 or 10 mg of gluten while performing the gluten contamination elimination diet (GCED) [23]. Doses of 5 and 10 mg are tiny amounts of gluten that are still tolerable and may be found in a standard GFD [5].

Outcome

The primary outcome of this study was the correlation between the amount of ingested gluten and the quantity of GIP excreted in urine during the following 24 hours.

Ethical clearance

This study was conducted in accordance with the principles of the Helsinki Declaration as revised in Fortaleza 2013 and was approved by the Ethical Committee of the Polytechnic University of Marche, Ancona, Italy (ID #131530). The trial was registered in the clinicaltrials.gov registry (ClinicalTrials.gov ID #NCT04477239).

Randomization

Randomization was performed using a random sequence generator (Research Randomizer, Version 4.0; https://www.randomizer.org/).

Dietary interventions

In study A, the GFD (see GFD Protocol, Supplementary Material 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A702) included commercially labeled and certified gluten-free food, that is, items containing less than 20 mg/kg (20 parts per million = ppm) of gluten (e.g., gluten-free bread, pasta, pizza, and flour) ensuring a daily gluten intake of ≤10 mg gluten per day, according to international regulations.

In study B, the GCED was designed to eliminate any possible source of gluten exposure, including the minute gluten traces (<20 ppm) that are allowed in a standard GFD [23]. To achieve the elimination of any possible source of gluten in the diet, almost all processed foods, even those foods labeled gluten-free, were removed; only whole, fresh unprocessed foods were allowed. As for cereals, only rice was allowed. The GCED scheme is shown in Supplementary Material 2, Supplementary Digital Content 2, http://links.lww.com/CTG/A703.

All the dietary schemes were administered by a dietitian with expertise in the treatment of CD. All participants were medical doctors with background knowledge of CD and of the GFD. They

were further educated about GFD restrictions. Participants were required to report each food/meal consumed during the 3 days of GFD in a food diary. The study was conducted during a period of time characterized by severe restrictions in dining out imposed by the coronavirus disease 2019 pandemic, a circumstance that facilitated the participants' adherence to the dietary regimens of the study.

Urine sampling and storage

All participants were provided with sterile containers and tubes for urine collection. For each gluten challenge, 3–5 mL urine samples were taken: (i) at baseline (after 3 days of GFD or GCED diet) and after the gluten challenge, (ii) from urine collected between T0 and T9, and (iii) from urine collected between T10 and T24. Volunteers were asked to keep the urine container at 4 °C and to record the volume of the T0-T9 and T10-T24 urine collections. The 5 mL aliquots were stored at −20 °C until delivered to the laboratory for analysis.

Quantification of GIP in urine samples

All laboratory tests were performed at the Celiac Disease Research Laboratory, Polytechnic University of Marche, Ancona, Italy. Urine GIP concentration was determined using the rapid immunochromatographic assay based on anti-gliadin 33-mer G12 monoclonal antibodies iVYCHECK GIP Urine test (In Vitro Diagnostics, Biomedal, Spain), according to the manufacturer's instructions. Samples showing a nonquantifiable readout (indicating the presence of 2.2–6.3 ng GIP/mL urine) were approximated to 4 ng/mL in the calculations below. The urinary GIP excretion was expressed as ng/mL and as ng/24 hours on the total volume of urine collected during the 24 hours after the challenge.

Determination of serum IgA anti-tissue transglutaminase antibody

IgA anti-tissue transglutaminase antibody assay was performed in our laboratory by a fluorescence enzyme immunoassay ≤30 days before the start of the study (normal values < 7 U/mL).

Statistical analysis

Sample size was calculated considering a repeated measures analysis of variance model using the expected difference in the mean urinary GIP excretion after gluten challenge and zero-gluten challenge as the primary response variable. Demographic data are presented as mean and SD or median and interquartile range (first–third quartiles) for the quantitative variables or absolute frequencies and percentages for the qualitative variables. The Shapiro-Wilk test was used to assess the normal distribution of the variables. The Wilcoxon signed-rank test was used to compare continuous variables. The $\chi 2$ test for trend was used to test the frequency equality of positive results on 6 baseline assessment challenges. Spearman's correlation coefficients and 95% confidence interval (CI) were used to estimate correlation between urinary GIP concentration evaluated at T0–T9 and T10-T24. A linear regression model with mixed random effects, which defined the subject as a random factor, was used to estimate the association between urinary GIP recovery in 24 hours (ng/24 hours) and the 6 increasing doses of gluten consumption (mg). In the regression framework, the GIP concentration acted as a dependent variable determined by the doses of gluten transformed on a logarithmic scale. Regression coefficients were estimated by 90% CI. The receiver operating characteristics (ROC) analysis was performed to evaluate sensitivity and specificity of urinary GIP, considering dose 0 mg (zero-gluten challenge) of gluten as the reference dose. The results are showed graphically reporting the observed and the estimated ROC curve with 90% confidence bands. Area under curve (AUC) and 90% CI were also estimated. Statistical analysis was performed using R software (version 4.0.2, 2019; R Core Team, Vienna; Austria), IBM SPSS Statistic v.23.0 (SPSS, Chicago, IL), and Microsoft Excel (v.2010; Microsoft Corp Redmond, Washington, DC).

1.3. RESULTS

Participants

Forty-five residents were eligible for participation; 25 accepted to participate, and all completed the study between October 2020 and February 2021. There were 21 women (84%) and 4 men

(16%), reflecting the higher female prevalence among residents in pediatrics, with a mean age of 31 years (SD 2, age range: 26–33 years). All participants showed a normal result of the serum IgA anti-transglutaminase determination.

Study A

Dietary compliance

Based on the analysis of the 24-hour food diary, no participant reported transgression to the GFD except one who inadvertently violated the protocol by tasting a gluten-containing cake during challenge n.4. The 2 urine samples after this challenge were eliminated from further analysis.

Urinary GIP determinations

Overall, 448 urinary samples were analyzed, 150 baseline (6 tests for 25 participants) and 298 after challenge (2 samples—T0–T9 and T10–T24—for 149 challenge procedures).

Figure 2 shows the results of the baseline urinary GIP determinations.

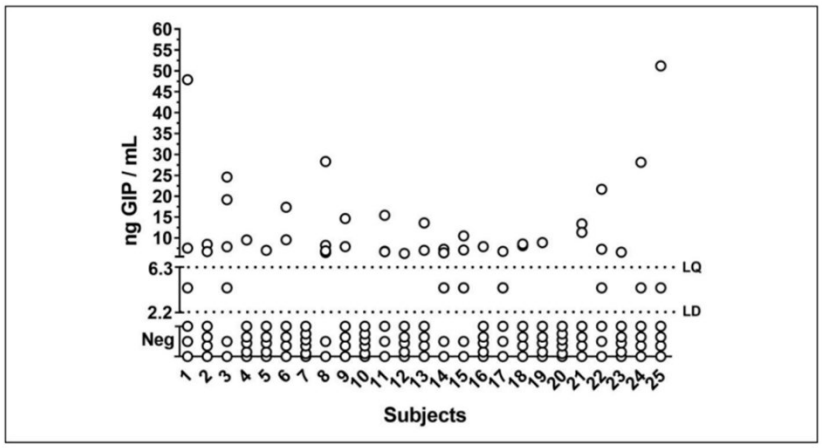

Figure 2. Baseline (T_0) urinary gluten immunogenic peptides determinations after 3 days of standard gluten-free diet. GIP, gluten immunogenic peptides; LD, limit of detection; LQ, limit of quantification.

Fifty-one of 150 baseline urine samples (34%) were positive for GIP, 40 (27%) with a quantifiable readout (median 8.21 ng/mL, range 6.30–51.18), and 11 (7%) below the quantification limit. No significant trend in the frequency of GIP+ baseline samples was observed from the first to the sixth challenge (36, 40, 28, 36, 36, and 28, respectively; P = 0.579).

As for postchallenge samples, no significant difference was detected in the distribution of urinary GIP concentration between T0-T9 and T10-T24 collections for each dose of gluten, and no significant correlation was found (Table 1).

Table 1. Comparison of gluten immunogenic peptides concentration (ng/mL) between 0–9 and 10–24 hours of urine collections

Gluten dose (mg)	T_0–T_9 collection Median (IQR)	T_{10}–T_{24} collection Median (IQR)	*P*	r (95%CI)
0	0 (0–4)	0 (0–6)	0.831	0.21 (−0.20; 0.56)
10	4 (0–7)	4 (0–14)	0.521	−0.05 (−0.45; 0.36)
50	0 (0–4)	0 (0–7)	0.357	0.26 (−0.15; 0.59)
100	0 (0–0)	4 (0–10)	0.052	−0.04 (−0.43; 0.36)
500	4 (0–7)	4 (0–11)	0.241	0.09 (−0.31; 0.47)
1,000	8 (0–16)	0 (0–7)	0.066	0.04 (−0.37; 0.44)

CI, confidence interval; IQR, interquartile range; r, Spearman correlation coefficient.
P value refers to the Wilcoxon signed-rank sum test.

After excluding urine samples collected from GIP+ participants at baseline, there were 7 of the 17 participants (41%) with GIP+ urine samples after the zero-gluten challenge, 4 (24%) of them on both T0–T9 and T10–T24 samples, 1 (6%) on T0–T9 only, and 2 (12%) on T10–T24 only. After the gluten challenge (10–1,000 mg), 55 of 81 urine samples (68%) showed urinary GIP positivity, 18 of 55 on T0–T9 only, 15 of 55 on T10–T24 only, and 22 on both T0–T9 and T10–T24 samples. In detail, 15 of the 18 participants (83%) showed T0–T9 and/or T10–T24 GIP+ urine samples after challenge with 10 mg, 12 of 20 (60%) with 50 mg, 10 of 19 (53%) with 100 mg, 10 of 13 (77%) with 500 mg, and 8 of 11 (73%) with 1,000 mg of gluten.

Dose/response relationship

Figure 3 shows the 24-hour urinary GIP recovery after each challenge procedure, expressed as ng/24 hours. Figure 4 shows the comparison between the zero-gluten challenge and the gluten challenge responses for each gluten level, after exclusion of all samples belonging to the baseline GIP+ subjects.

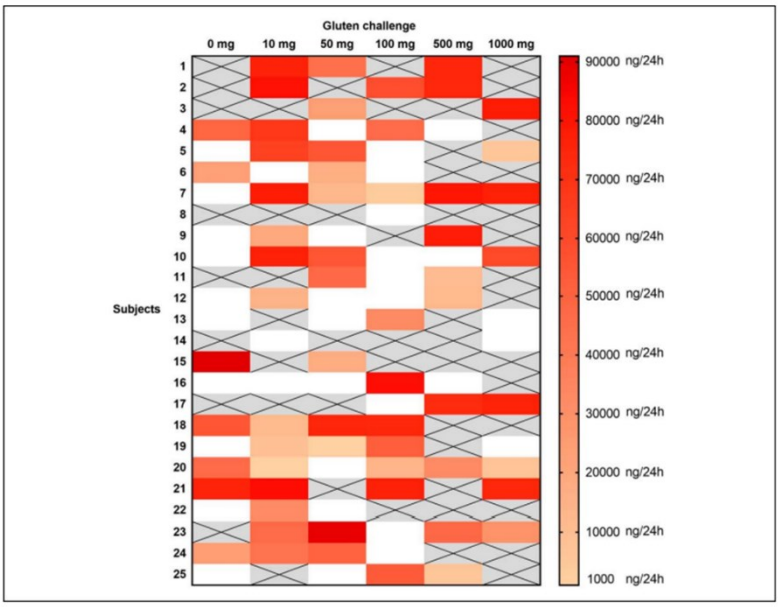

Figure 3. Results of the 24-hour urinary GIP determination after the different gluten challenges in subjects performing the standard gluten-free diet. Crossed gray cells: samples excluded because of a positive T0 result. White cells: GIP-negative samples. GIP, gluten immunogenic peptides.

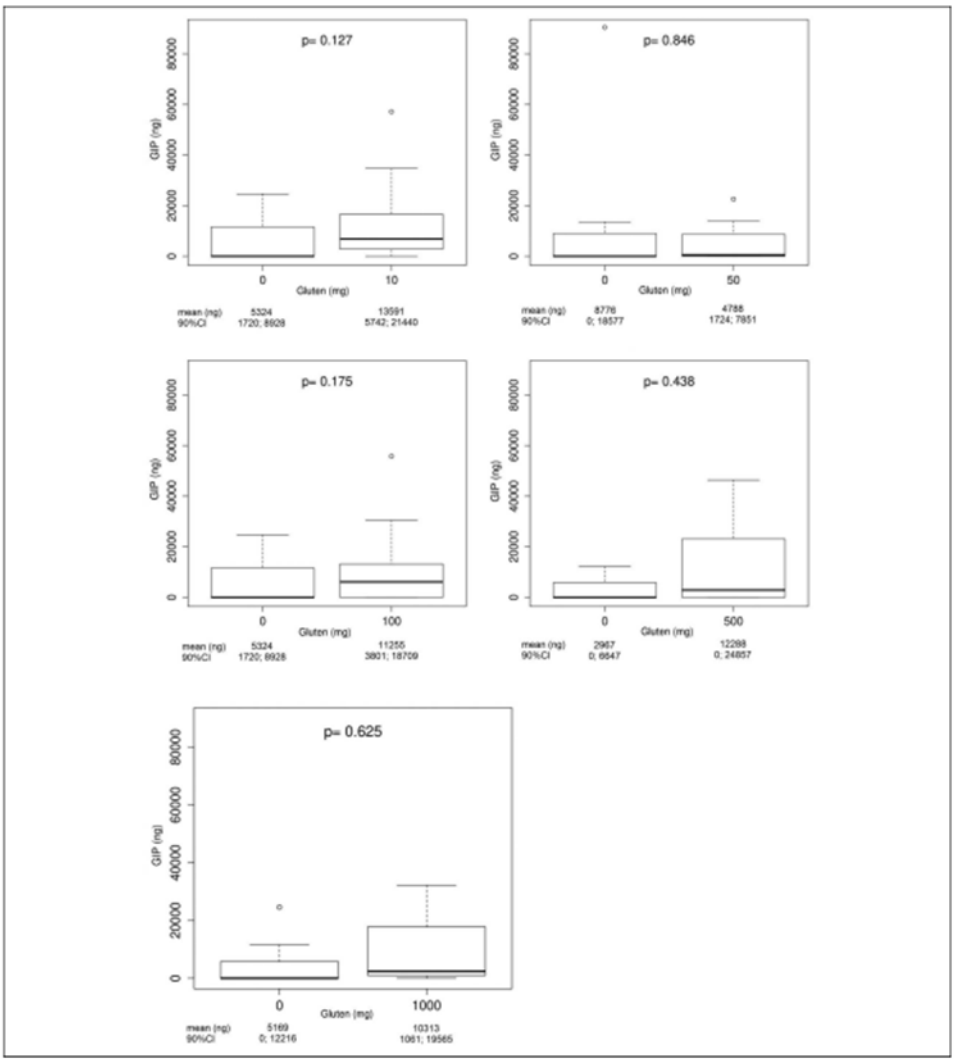

Figure 4. Comparison between the zero-gluten challenge and the gluten challenge urinary GIPs responses at different levels of gluten intake. *P* values refer to the Wilcoxon signed-rank test. GIP, gluten immunogenic peptides.

There was no significant difference between the zero-gluten challenge and the gluten challenge response for all doses of gluten. The regression coefficient estimated that the mixed-effect linear model was equal to 96 (95% CI = −518; 709), showing no significant change in urinary GIP content when the gluten dose increased from 0 to 1,000 mg. Figure5 shows the results of the ROC analysis, considering the zero-gluten dose as reference. Doses 10, 500, and 1,000 mg had AUC values between 0.67 and 0.69, with the lower limits of 90% CIs close to 0.50. Doses 50 and 100 mg showed observed AUC values close to 0.50, with the observed and estimated ROC values very close to the diagonal of the graph.

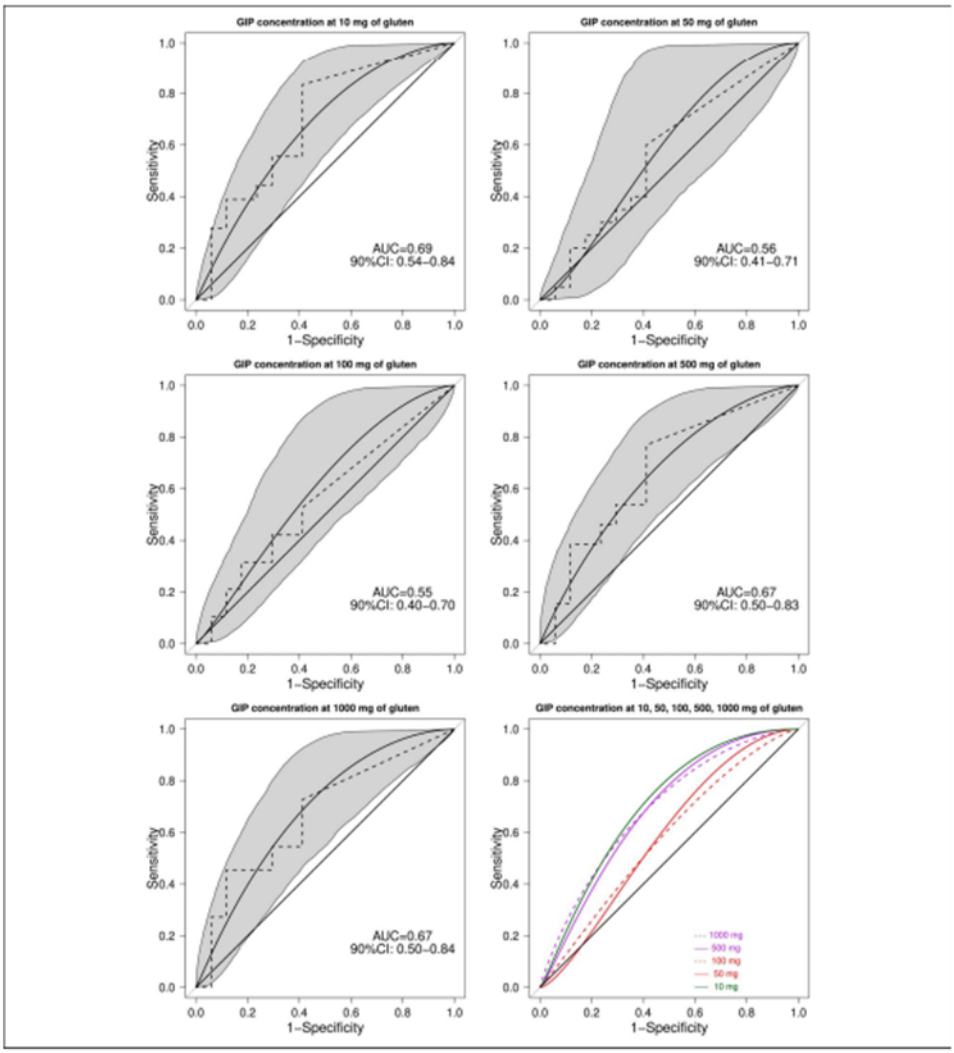

Figure 5. Observed (dotted line) and estimated ROC curves (solid line) with 90% confidence bands (gray area). AUC, area under curve; CI, confidence interval; ROC, receiver operating characteristics.

Study B

Dietary compliance

Based on the analysis of the 24-hour food diary, no participant reported transgression to the GCED.

Urinary GIP determinations

After 3 days of GCED, baseline urine samples (n = 24) constantly tested negative for GIP (Figures 6 a,b). After the challenge with microdoses of gluten, 8 of 24 (33%) showed GIP positivity.

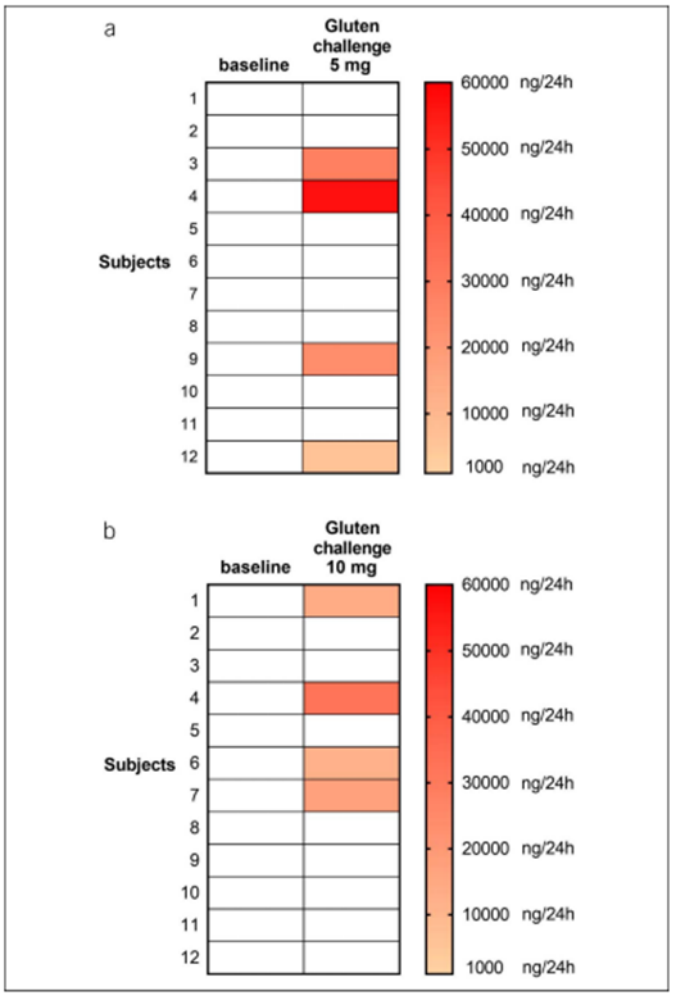

Figure 6. Results of 24-hour urinary GIP determination before and after the 5 mg (**a**) and the 10 mg (**b**) gluten challenge in subjects performing the gluten contamination elimination diet. White cells: GIP-negative samples. GIP, gluten immunogenic peptides.

In detail, 3 of 12 participants were GIP positive after taking 5 mg of gluten (2 only on the T0–T9 sample and 1 on both T0–T9 and T10–T24 samples), 3 of 12 were positive after taking 10 mg (2 only on the T9 sample and 1 on both T9 and T24 samples), and 1 was positive at both doses, on both T0–T9 and T10–T24 samples after 5 mg and on the T0–T9 sample after 10 mg of gluten.

1.4. DISCUSSION

In a group of healthy and qualified volunteers undergoing dietary challenges with increasing amounts of gluten, the performance of urinary GIP determination in monitoring the GFD was poor. Indeed, a significant percentage of subjects had a positive GIP determination on a strict GFD (34%) and/or after the zero-gluten challenge (41%). At the same time, a high percentage of subjects had a negative GIP determination after challenges with a significant amount of gluten (up to 1 g). In the past few years, a growing interest has focused on the assessment of compliance to the GFD, and GIP determination in stools or urine has been the most promising tool (12–22). GIP are fragments of gluten proteins that are reactive to the anti–33-mer G12 monoclonal antibody. A small fraction of ingested gluten peptides is either adsorbed and excreted in urine or excreted in stools, thereby revealing ongoing gluten exposure. Fecal GIP positivity has been found in 16%–30% of treated patients with CD [8,10,12,13]. In a systematic review, the GIP assay showed the lowest celiac dietary adherence rate (75%) in children with CD on a GFD as compared with the intestinal biopsy (87%), self-report (81%), structured dietary interview (77%), and CD serological markers (76%), suggesting that this test is more sensitive than other methods of GFD monitoring [6]. Healing of the small intestinal mucosa has been associated with the repeated absence of urinary GIP in treated patients with CD [7,21]. However, previous studies assumed that the absence or presence of GIP in urine directly reflects absence or presence of contaminating gluten in the GFD, an axiom that has never been investigated in depth. As for the dose/response relationship, the only available data showed that 3/4 and 4/4 out of 4 healthy subjects kept on the GFD had at least 1 positive

urinary GIP test after a challenge with 25 or 50 mg of gluten, respectively [11]. It should be noted that 25 mg is a tiny amount of gluten, close to the maximum amount that is tolerable in the standard GFD, that is, 10 mg/d [5,24].

Our study evaluated the performance of urinary GIP determination in a randomized, double-blind, controlled gluten challenge trial for the first time. We found that 34% of healthy subjects showed a positive urinary GIP test after a strictly controlled standard GFD for 3 days. After excluding subjects with GIP positivity at baseline, 41% had a positive GIP determination after a zero dose of gluten. At first glance, these disappointing results could be explained by (i) prolonged urinary GIP elimination (≥3 days) after stopping the gluten-containing diet, (ii) overestimation of false positives secondary to testing urine collections instead of random urine samples, and (iii) poor compliance with the GFD. Despite many precautions taken to avoid dietary transgression (the strong motivation of participants and their professional awareness of the GFD requirements, in-depth monitoring of the diet, and impossibility to dine out because of coronavirus disease 2019–related restrictions), we cannot exclude dietary mistakes in participants who followed the GFD for only 72 hours. However, the results of study B suggest a different and more convincing explanation.

After 3 days of the GCED, all urinary samples were indeed GIP-negative, whereas a challenge with minute amounts of gluten (5 or 10 mg) was sufficient to cause positivity of urinary GIP in 33% of cases. In other words, a true zero-gluten diet (the GCED) was constantly associated with a negative urinary GIP test, whereas the traces of gluten that may be found in commercially available gluten-free food (that may generate an intake of up to 10 mg/d of gluten by definition) yielded a positive result in a significant proportion of cases. Therefore, the urinary GIP test seems to be somewhat too sensitive and may result positive even in subjects perfectly complying with the requirements of the standard GFD. These findings have an important clinical implication. The previously reported high rate of positive GIP tests in patients with CD on a GFD should not be interpreted as evidence of poor compliance to the GFD, an issue that has raised many concerns in the real life of celiac patients. On the other hand, this procedure

might find application in the monitoring of hypersensitive patients with CD treated by the GCED.

Our study disclosed further limitations of the urinary GIP test, first the high percentage (25%) of negative urinary GIP results after macrodoses of gluten (500–1,000 mg). Whether the consequence of the complete digestion of GIP into the gut, for example, related to a specific proteolytic activity of the intestinal microbiota [9], or whether caused by some other unknown factor, for example, an abnormal intestinal transit time, this result indicates that the negative predictive value of the test is poor (Figure 5). Another still unclear issue is the kinetics of urinary GIP elimination. It was originally suggested that GIP are detectable in urine only between 3 and 9 hours from gluten ingestion. However, we found a similar number of postgluten challenge urinary GIP positives in the T0–T9 and the T10–T24 urine collections, suggesting that delayed GIP elimination is common. Finally, we did not find any dose/effect relationship between the quantity of ingested gluten and the amount of urinary GIP (Figure (Figure3). All these findings suggest that the urinary GIP test may not be an effective tool for monitoring a GFD adherence and seriously dispute the validity of studies estimating the amount of contaminating gluten in a diet through the application of a complex (and largely theoretical) conversion factor to the concentration of GIP in a random urine sample [15]. On the other hand, the diagnostic accuracy of stool GIP determination remains to be evaluated.

The strengths of this study are the accuracy of the study design, the reliability of participants, the standardization of the gluten challenge, and the large number of challenge procedures. The weaknesses are the small size of the study group, the impossibility to fully control the complete adherence to the GFD in a real-life scenario, and the use of raw (rather than cooked) gluten for the challenge procedures. It also remains to be clarified whether our findings may extend to patients with CD, although the standardization of the urinary GIP test in patients frequently showing a variable degree of intestinal mucosa damage, as it is the case in subjects with treated CD [25], could prove even more difficult than in healthy controls.

In conclusion, this study suggests that the urinary GIP determination may not be an accurate method to assess the adherence to the standard GFD, but the test may find application in

monitoring a zero-gluten diet as the GCED. Additional validation studies are needed to investigate the diagnostic accuracy and the dose/response of GIP determination in stool.

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Chapter 5 **– Slow Decrease of Antitissue Transglutaminase Antibody Positivity in Children With Celiac Disease After Starting the Gluten-free Diet *(JPGN 2020)***

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Letter to the Editor

Sansotta et al. [1] reported on the analytical performance of 2 different assays in monitoring IgA antitissue transglutaminase antibody (anti-tTG) normalization in children with celiac disease (CD) after starting treatment with the gluten-free diet (GFD). The authors reported that anti-tTG levels were still abnormal after 30 months of GFD in a significant proportion of cases (14%–30%). They also reported a longer median time of anti-tTG normalization by the chemiluminescence immune assay (CLIA) compared with enzyme-linked immunosorbent assay (ELISA). We retrospectively analyzed 54 consecutive CD children diagnosed during 2017 who presented for follow-up visits in our Celiac Clinic. Anti-tTG antibodies were assayed by fluorescence enzyme immunoassay (FEIA), a different technique. Similar to the results by Sansotta et al. [1], we found that 49%, 32%, and 15% of cases still showed a positive anti-tTG level at 6, 12, and 24 months, respectively. By binary logistic regression analysis, the risk of antitTG positivity after 12 months of GFD was influenced as follows: 20% increased risk for each additional year of age at diagnosis; 10% increased risk for each additional 1 of IgA anti-tTG level at diagnosis; and 11 higher risk in girls compared with boys. Consistent with the data of Sansotta et al and previous pediatric studies [1–4], we confirm that: a significant proportion of CD children show a slow decrease of anti-tTG levels after starting the GFD, with persistent antibody positivity after 24 months of the GFD. During this period, that may persist for 4 years

[3], the diagnostic significance of IgA anti-tTG is problematic; because of the variable performance of different techniques for anti-tTG determination, it is advisable to use the same analytical technique for CD follow-up monitoring.

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Chapter 6 **– Nutritional Status, Dietary Intake, and Adherence to the Mediterranean Diet of Children with Celiac Disease on a Gluten-Free Diet: A Case-Control Prospective Study *(Nutrients 2020)***

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1.1. BACKGROUND

Celiac disease (CD) is a systemic immune-mediated disorder caused in genetically susceptible persons by the ingestion of gluten-containing grains [1]. The only available treatment is the gluten-free diet (GFD), which consists of the dietary exclusion of grains containing gluten (i.e., wheat, rye, barley) [2].

The nutritional adequacy of the GFD remained controversial and a matter of debate for a long time [3]. Indeed, apart from maintaining the safe limit of gluten intake (below 10–50 mg/day), a suitable GFD must also be nutritionally balanced and cover all energy and nutrient requirements to prevent deficiencies and ensure a healthy life. In children, the GFD must also allow appropriate growth and pubertal development [4]. A body of evidence has so far suggested that the GFD may be nutritionally unbalanced either because of the need to exclude several cereals or because of the different nutritional composition of GF products as compared to their unrefined analogs [3,5,6,7,8,9,10,11,12,13,14,15,16].

To the best of our knowledge, there are no large case-control studies performed on children regarding the nutritional adequacy of the GFD. Previous studies have mostly been performed on adolescents or adults, with the limit of (1) small sample sizes, (2) lack of a control group, (3) retrospective methods of dietary recording, and (4) inclusion of patients at diagnosis.

Therefore, we aimed to evaluate the nutritional status, the dietary intake and adherence to the national recommended dietary allowances as well as to the Mediterranean diet of Italian children with CD on the GFD by a large, prospective case-control study.

1.2. METHODS

Study Population

This is a case-control prospective study conducted at the Center for Celiac Disease of the Polytechnic University of Marche from January 2017 to January 2019. All children (age range = 4–16 years) with a diagnosis of CD according to the ESPGHAN criteria [17], on a GFD for ≥ 2 years, were recruited as the CD-group. Patients who (1) had other chronic conditions (including type 1 diabetes or inflammatory bowel disease) or (2) did not adhere to the GFD (as demonstrated by elevation of serologic CD markers at enrollment) were excluded. Controls were healthy age- and gender-matched children not affected with CD (on the basis of a negative result of the IgA class anti-transglutaminase test), participating in a previously described mass screening program for CD [18]. Children with comorbidities or following a special diet for other reasons (vegetarian, vegan diet, or related to particular religious or social traditions) were excluded.

Anthropometric Measurements

For all children, anthropometric measurements were collected by the same trained operator. Body weight was measured using the same mechanical balance (mod. 200, SECA, Limbiate, Italy); height was measured to the nearest 5 mm using a stadiometer (mod. 220, SECA, Limbiate, Italy). Body mass Index (BMI) was calculated from weight and height (Kg/m2). The BMI values were categorized according to the World Health Organization criteria as follows: below 18.5 kg/m2 considered as underweight, 18.5–24.9 kg/m2 as normal weight, 25–29.9 kg/m2 as overweight and >30 kg/m2 as obese.

Physical Activity

For all children, information about lifestyle, such as the number of weekly hours devoted to physical activity and number of daily hours devoted to sedentary activities (e.g., sitting down in front of the TV, PC, tablet, PlayStation or board games) were collected by a detailed questionnaire.

Dietary Assessment

In both groups, dietary intake was assessed using a 3-day food diary, two on weekdays and one at the weekend. The diary was carefully explained by the same trained dietitian to both children and their parents and was accompanied by detailed instructions for the compilation and a photographic atlas including different portion-size food pictures and a set of about 60 actual household measures. The diary was specifically developed for CD patients and included a daily record of all foods consumed during the different meals (breakfast, morning snack, lunch, afternoon snack, dinner). For each meal, participants were requested to report an exhaustive description of food and recipes (including cooking and preservation methods, sugar or fats added during meal preparation), food amount (according to the atlas) and brand of packaged foods consumed.

All diaries were analyzed by the same trained dietician using an Excel spreadsheet (specifically developed for the study) to estimate the composition of the macronutrients of the diet and the frequency of foods. In the database each consumed food was classified into the main food group categories: sugary drinks, meat, processed meat, vegetables, fruit, milk and dairy products, legumes, potatoes, fish, eggs and cereals (including pasta, bread and bakery products, rice, minor cereals—e.g. oats- and pseudo-cereals—e.g. buckwheat and quinoa), sweets and salty snacks. Each food group had several subgroups (i.e., cereals had 5 subgroups: pasta, bread products, pizza, rice, minor cereals and pseudo-cereals), and each subgroup was further classified according to its composition (i.e., bread was divided in whole grain bread, type 0 flour bread, type 00 flour bread, milk bread, durum wheat bread, rye bread, etc.), allowing us to estimate the different nutritional composition of each consumed food. The

source of information of the nutritional composition of foods was the Italian Food Composition Database [19]. The composition of GF products was retrieved from product labels. Weight of consumed foods was calculated based on the weight of raw foods, as recommended [19]. By using portion size photos, the weight of the portion for different foods was obtained by the guidelines of the Italian Society of Human nutrition [20]. In the presence of several ingredients, we included in the database only foods reaching the size of a portion.

The program estimated the energy intake (Kilocalories), and macronutrients (proteins, total fats, saturated fast, carbohydrates, simple sugars, and fiber—expressed in grams) and the percentage of energy provided by each macronutrient. “National Recommended Energy and Nutrient Intake Levels” (LARN) issued by the Italian Society of Human Nutrition in 2014 [20] and the “Italian Food Pyramid” (IFP) recommended by the Italian Society of Pediatrics [21] were taken as reference values for energy and nutrient intake and for food group consumption, respectively. For CD patients, the impact of commercial GF products specifically formulated for CD in terms of energy and macronutrients was also estimated.

The adherence to the Mediterranean diet was estimated by the KIDMED index (Mediterranean Diet Quality Index in Children and Adolescents) [22], widely used as an indicator of healthy dietary habits. This index is determined from a 16-point questionnaire that assesses various dietary habits. Each answer is scored according to whether it is consistent with habits associated with the Mediterranean pattern, and scores are added up to quantify the total index of the subject’s adherence to the Mediterranean diet (MD). The KIDMED index ranges from 4 (no adherence to the MD) to 12 (complete adherence to the MD) [22]. The KIDMED test and scoring is attached as Supplementary Table S1. The study protocol was approved by the Institutional Review Board of the Polytechnic University of Marche (Ancona, Italy). All subjects gave their informed consent for inclusion before they participated in the study.

Statistical Analysis

Based on a previous pediatric study [23] and our preliminary data, considering an expected mean BMI of 16 in healthy children, and a mean BMI of 17 in CD children, with a level of

significance of 0.05 and a power of 90%, a minimum sample of 95 CD children was calculated. Subjects' general characteristics were summarized using descriptive statistics: median, first and third quartiles for quantitative variables, and absolute and percent frequencies for qualitative variables. Comparisons between the two groups were performed by means of the Wilcoxon rank-sum test and Fisher test, respectively. The comparison between the estimated levels of energy, macronutrients and food groups consumption references values (LARN and IFP) was carried out using the 95% Confidence Interval (95% CI) for the median. A probability of 0.05 was chosen to assess the statistical significance; the R program (Institute of Statistics and Mathematics, Vienna, Austria) was used for statistical analysis.

1.3. RESULTS

Study Population

The net participation rate was 90%. The main reasons for refusal were lack of time, no interest, and difficulty to reach the study center (Figure 1). Overall, 120 CD children were enrolled; there were 72 females (60%), the median age was 10.5 (range: 4.4–15.5 years), with a median duration of GFD of 2.6 years (first and third quartiles, 1.4–4.3 years). The control group included 100 healthy children, 56 females (56%), with a median age of 10.1 (range: 4.7–14.5 years).

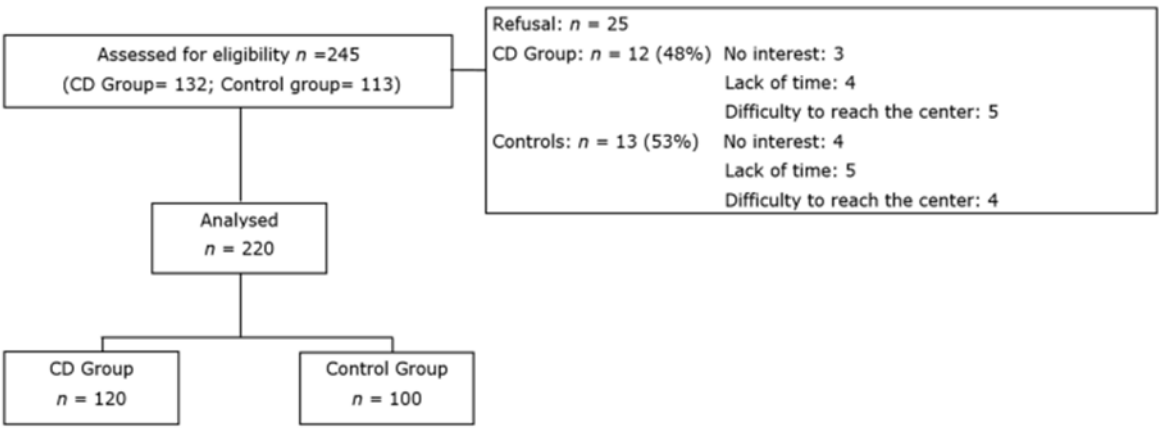

Figure 1. Flow diagram of the study.

Anthropometric Results and Energy Expenditure

As shown in Table 1, no differences were found between CD children and the control group as regards anthropometric measurements and energy expenditure. In detail, the median BMI was 16.8 in CD children and 16.0 in the control group, with no significant difference between the groups, and the prevalence of overweight and obesity was similar. The self-reported physical activity, as well as the number of daily hours devoted to sedentary activities, were comparable.

Table 1. Anthropometric characteristics and energy expenditure of CD children and control group.

Variable	CD Group (n = 120)	Control Group (n = 100)	p
Age (years)			
Median	10.5	10.1	0.494
First to third quartiles	8.3–12.2	9.4–13.5	
Weight (Kg)			
Median	31.5	32.5	0.448
First to third quartiles	25.2–42.0	28.2–50.0	
Height (cm)			
Median	1.4	1.4	0.26
First to third quartiles	1.2–1.5	1.4–1.5	
Body mass index (Kg/m^2)			
Median	16.8	16.0	0.988
First to third quartiles	15.3–18.9	15.2–19.5	
Body mass index class (Kg/m^2)			
Underweight-n (%)	6 (5)	5 (5)	
Adequate weight-n (%)	78 (65)	70 (70)	0.35
Overweight-n (%)	24 (20)	18 (18)	
Obese-n (%)	12 (10)	7 (7)	
Physical activity (hours/week)			
Median	4	4	0.729
First to third quartiles	3–8	4–5	
Sedentary activity (hours/week)			
Median	2	2	0.247
First to third quartiles	1–2	2–3	

Total Energy, Macronutrient Intakes and Adherence to LARN

Table 2 shows the total daily energy and the macronutrient intakes in the two study groups, and the comparison with the LARN recommendations.

Table 2. Daily intake of energy, macronutrients of CD children and the control group, and comparison with National Recommended Energy and Nutrient Intake Levels (LARN).

Group		CD Group (n = 120) Median (1st; 3rd Quartiles) 95% Confidence Interval	Control group (n = 100) Median (1st; 3rd Quartiles) 95% Confidence Interval	p	LARN
Energy	Kcal	1819.3 (1589.6; 1997.0)	1838.2 (1782.2; 1964.6)	0.225	
		1715.5–1923.0	1775.3–1901.1		
Total Proteins	grams	59.4 (53.4; 67.7)	65.9 (60.7; 72.0)	0.095	
		55.7–63.0	62.0–69.8		31–62 (PRI)
	% Energy	13.4 (11.8; 14.4)	14.1 (12.4; 14.7)	0.335	
		12.7–14.0	13.4–14.9		10–15% (RI)
	grams/Kg	1.9 (1.5; 2.6)	1.9 (1.7; 2.2)	0.670	
		1.6–2.2	1.7–2.1		0.90–0.99 (PRI)
Total Carbohydrates	grams	209.7 (184.1; 252.1)	260.5 (245.4; 298.4)	**0.001 ***	
		192.5–226.8	242.2–278.8		
	% Energy	46.9 (42.6; 51.7)	53 (50.5; 56.8)	**0.001 ***	
		44.5–49.2	50.9–55.2		45–60% (RI)
Total sugars	grams	68.1 (49.1; 83.3)	83.1 (69.7; 95.3)	**0.036 ***	
		59.4–76.9	74.2–91.9		
	% Energy	14.5 (10.4; 17.6)	17.6 (14.2; 19.6)	**0.036 ***	
		12.6–16.3	15.8–19.4		<15% (SDT)
Total fats	grams	78.1 (63.9; 92.2)	64.4 (59.5; 74.4)	**0.015 ***	
		70.8–85.4	59.2–69.5		
	% Energy	37.5 (32.8; 40.5)	30.5 (28.7; 32.3)	**0.001 ***	
		35.6–39.5	29.3–31.8		20–35% (RI)
Saturated fats	grams	25.3 (20.2; 30.8)	18.7 (16.5; 21.7)	**0.003 ***	
		22.5–28.1	16.9–20.4		
	% Energy	12.8 (10; 14.7)	8.8 (7.8; 10.4)	**0.001 ***	
		11.6–14.0	7.9–9.7		<10% (SDT)
Total Fiber	grams	12.6 (10.9; 16.7)	15 (13.5; 19.1)	**0.015 ***	
		11.1–14.2	13.1–16.9		
	% Energy	1.4 (1.1; 1.7)	1.7 (1.4; 1.9)	0.067	
		1.2–1.5	1.5–1.9		At least 1.7% (AI)

PRI: Population Reference Intake; RI: Reference Intake range for macronutrients; SDT: Suggested Dietary Target; AI: adequate intake. *: Statistically significant

The estimate of daily energy intake was similar in the two groups. Protein consumption did not differ between CD and control children, and both the daily protein intake and the daily energy intake provided by proteins were in line with the LARN recommendations in both groups. The daily intake of carbohydrates and the energy intake provided by carbohydrates were significantly lower in the CD group (209.7 g in the CD group versus 260.5 g in the control group; p = 0.001), although in both groups the percentage of energy supplied by carbohydrates reached the LARN recommendations. The daily intake of simple sugars and their contribution to the daily energy intake were significantly different between the two groups, with a higher intake in the control group. Furthermore, the daily intake of simple sugars exceeded the LARN recommendations (<15% of total energy) only in the control group.

The daily intake of total fats and saturated fats were significantly higher in the CD group (total fats: 78.1 g in the CD group versus 64.4 g in the control group; p = 0.015; saturated fats: 25.3 g in the CD group versus 18.7 g in the control group; p = 0.003). Indeed, the energy intake provided by total fats and saturated fats was significantly higher in the CD group and exceeded the nutritional goal recommended by LARN (<10% Total Energy). Finally, the daily consumption of fiber was significantly different in the two groups, with a lower daily intake in the CD group (12.6 g in the CD group versus 15 g in the control group; p = 0.015); moreover, the energy intake provided by fibers was lower as compared to the appropriate intake suggested by LARN (at least 1.7%), while in healthy controls it reached the lower normal limit.

Food Group Intake and Adherence to IFP

Table 3 shows daily food group consumption, as collected by the 3-day food diary. CD children showed a higher consumption of processed meat and salty snacks as compared to healthy children (2.5 portions in the CD group vs 2 in the control group; p = 0.009, and 1 portion vs. 0; p = 0.001, respectively).

Both groups did not reach the number of portions recommended by the IFP for legumes, vegetables, eggs, and fish, while exceeding in the consumption of sugary drinks, meat and processed meat. The consumption of cereals, milk and dairy products and potatoes reached the IFP recommendations in both groups; in the group of cereals, the consumption of pseudo-cereals was very low in the CD group, and the major contributors were GF products specifically formulated for CD. The consumption of fruit reached the minimum intake recommended by the IFP.

Table 3. Food group consumption in CD children and the control group, and comparison with the recommendations of the Italian Food Pyramid. Consumption is expressed as median of portions consumed over 3 days of recording.

Food Groups	CD Group (n = 120)	Control Group (n = 100)		
	Median (1; 3 Quartiles)	Median (1; 3 Quartiles)	p	IFP
	IC 95%	IC 95%		
Sugary drinks	1 (0; 2)	2 (1; 2)	0.401	
	0.5–1.5	1.5–2.5		Lowest consumption
Meat	2 (1; 3)	2 (1; 3)	0.831	
	1.5–2.5	1.5–2.5		1
Processed Meat	2.5 (2; 4)	2 (1; 2)	**0.009** *	
	2.0–3.0	1.5–2.5		1
Cheese	1 (0; 3)	1 (0; 2)	0.431	
	0.5–2.0	0.5–1.5		1
Fruits	2.5 (1; 4)	3 (2; 5)	0.121	
	1.5–3.0	2.0–4.0		3–6
Milk and yogurt	3 (1; 3)	3 (3; 4)	0.116	
	2.5–3.5	2.5–3.5		3–6
Legumes	0 (0; 0)	0 (0; 1)	0.175	
	0–0	0–0.5		2
Potatoes	1 (0; 1)	1 (0; 1)	0.475	
	0.5–1.0	0.5–1.5		1
Fish	1 (0; 1)	1 (0; 2)	0.121	
	0.5–1.0	0.5–1.5		1–2
Eggs	0 (0; 1)	0 (0; 1)	0.569	
	0.0–0.5	0.0–0.5		1
Vegetables	2.5 (1; 4)	3 (2;4)	0.122	
	1.5–3.0	2.5–3.5		6–9
Total cereals	15.5 (13; 17)	15 (14; 18.5)	0.463	
	14.5–16.5	13.5–16.5		9–15
Maize	0 (0; 0)	0 (0; 0)	0.906	
	0–0	0–0		Not indicated
Rice	1 (0; 1)	1 (0; 1)	1	
	0.5–1.0	0.5–1.5		Not indicated
Minor and pseudo-cereals	0 (0; 0)	0 (0; 0)	0.167	
	0–0	0–0		Not indicated
Salty snaks	1 (0; 2)	0 (0; 0.5)	**0.001** *	Lowest consumption
	0.5–1.5	0–0.5		

*: Statistically significant.

Impact of Commercial GF Products Specifically Formulated for CD

Commercial gluten-free products specifically formulated for CD contributed to 73% of daily carbohydrates, 59% of fibers, 34% of sugars, 28% of total fats, 25% of saturated fats and 22% of proteins. Finally, commercial gluten-free products specifically formulated for CD provided 46% of the total daily energy.

KIDMED Index

The median KIDMED index was 6.5 in CD children and 6.8 in healthy controls, showing a suboptimal adherence to the Mediterranean diet in both groups.

1.4. DISCUSSION

The present case-control study shows that the nutritional status of CD children does not differ from healthy children. However, the diet of CD children in this study was nutritionally less balanced than controls, with a higher intake of fat and a lower intake of fiber, highlighting the need for dietary counseling.

Data from the literature on the effects of GFD on anthropometric parameters of patients with CD are controversial. On the one hand, it has been reported that a good compliance to the GFD is associated with a positive effect on anthropometric parameters with a recovery of lean body mass, normalization of BMI in both underweight and overweight children, and acceleration of linear growth [23,24,25,26]. On the other hand, there are also studies suggesting that the GFD may have a negative effect on body composition and anthropometric parameters in CD patients, with an increased prevalence of overweight and obesity [5,27,28]. These conflicting data may in part be caused by differences in the duration of the GFD at the time of anthropometric assessment or by the lack of a control group. Our study is the first to evaluate the BMI in a large sample of CD children on a GFD for at least two years as compared to healthy children, showing that there is no difference in the percentage of underweight, normal weight and overweight/obesity between groups. We also evaluated the energy expenditure in the two study groups through lifestyle analysis, showing no differences between CD children and the control group. The similar BMI in the presence of a similar lifestyle suggested that energy intake was similar in the two study groups. Indeed, we did not observe any difference in total daily energy intake.

Nonetheless, concern about the nutritional quality of the GFD emerges from our results. Indeed, by the analysis of 3-day food diaries, we found a higher intake of fat and a lower intake of fiber and carbohydrates in CD children on a GFD as compared to healthy children, while there was no difference in the daily intake of protein. As regards carbohydrates, when comparing the daily intake of macronutrients of CD and control children with the Italian recommendations, the percentage of energy supplied by carbohydrates was, however, in line

with the LARN recommendations in both groups. Noticeably, healthy children exceeded the daily intake of simple sugars as compared to LARN recommendations, while CD children did not. The main concern about GFD was the higher consumption of total and saturated fats observed in CD children, with the intake of saturated fat exceeding the nutritional goal recommended by LARN only in the CD group. The intake of fiber was also a concern, being lower in CD children as compared to controls and to LARN recommendations.

Our findings are in line with several previous studies that compared the intake of macronutrients in CD patients with the national recommendations, showing, overall, that CD patients consume less fiber and more fats than recommended [5,7,9,10,11,13]. When comparing the nutritional quality of CD patients on a GFD to that of healthy controls, previous studies showed conflicting results. Consistent with our findings, several studies in adults reported a higher intake of fats in CD patients as compared to healthy subjects [6,8,13], while others reported a lower intake of carbohydrates and protein [5] or only a lower intake of fiber [7] or no differences in CD adolescents as compared to a control group [12]. Finally, Zuccotti et al. showed a higher intake of carbohydrates and lower consumption of fat in 18 CD children as compared to 18 healthy controls by a 24 h recall [11]. Differences between studies may be explained by the different age of patients studied (children versus adolescents and adults), the small sample size of many previous studies, the different methods of dietary collection (prospective food diary versus retrospective recall), and finally by the inclusion of patients both at diagnosis and on GFD in some of the studies. Our study firstly evaluated prospectively the macronutrient intake in a large sample of CD patients of pediatric age with at least 2 years' experience of GFD as compared to healthy subjects by 3-day food diary that is one of the best-practice methods to obtain dietary data [29].

One of the main factors that could explain the unbalanced intakes of nutrients is the dietary pattern. For this reason, in our study, we compared the dietary habit of CD children and healthy subjects with respect to the IFP, showing that: (a) CD children have a higher consumption of processed meat and salty snacks as compared to healthy children; (b) both groups did not reach the portions recommended by the IFP for legumes, vegetables, eggs and fish, while

exceeding the consumption of sugary drinks, meat and processed meat; (c) the consumption of minor and pseudo-cereals was very low in the CD group, and the major contributors to cereals were gluten-free products. These results may explain the higher intake of fat and lower intake of fiber observed in CD children, however, they also highlight that the dietary habits of Italian children, either celiacs or controls, are not fully adherent to the Mediterranean diet. Indeed, the KIDMED index was moderate in both groups.

Furthermore, several studies have shown that the nutritional profile of GF products specifically formulated for CD patients is different with respect to regular foods, with a higher content of fat and saturated fat, salt, sugar and a lower content of fiber [14,15]. In our study, commercial GF products specifically formulated for CD patients provided 46% of the total daily energy, thus playing a major role in influencing the imbalance in the diet of CD children. Many GF foods are prepared from refined maize flour and white rice, which are lower in fiber (2.6 g and 0.7 g per 100 g, respectively) than wheat (3.5 g per 100 g) or whole wheat (9.6 g per 100 g) [19]. The exclusion of gluten and the use of only GF raw materials as ingredients result in GF food which is less palatable than regular foods; consequently, the manufacturing of GF foods requires the addition not only of some additives, such as hydrocolloids, but also of some macronutrients, such as fats in the final products to mitigate the loss of gluten. Our study highlights the need of enhancing the nutritional quality of GF products.

The main weakness of the present study was the lack of data on micronutrient intake, an important piece in the puzzle of the nutritional quality of the GFD. This limitation was related to the lack of tables on GF products indicating the micronutrient content. Therefore, it was not possible to accurately evaluate the corresponding intake in the diet. Second, potential recording errors including inaccurate estimates of portions consumed and omission of foods (either deliberate or unintentional) could result in an underestimation of nutritional intake, as in all food diary recording. Finally, results on food group intake were collected by the 3-day food diary, that is not the best instrument to estimate the consumption frequency of some foods, such as those that are not eaten daily (e.g., legumes, fish, egg).

1.5. CONCLUSIONS

Our study, together with a review of the literature, highlights the need for celiac patients to receive dietary counseling, a fundamental tool to teach the patient to increase the consumption of naturally gluten-free products, to reduce processed ones, to increase the intake of cereals such as oats, rice, minor and pseudo-cereals, and to adhere to the rules of the Mediterranean diet. Nonetheless, our study underlines the need for an adequate nutritional educational program and healthcare policies also for healthy children to ameliorate nutrient intake during childhood, possibly impacting on long-term health outcome.

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Chapter 7 **– The Celiac Disease-Children's activities report (CD-Chart): cross-cultural comparison in Italy and Israel.**

1.1. BACKGROUND

Managing the gluten free can be challenging for children with celiac disease in their daily life encounters and activities. The objective of this study was to compare characteristics of participation in food-related activities as measured by the Celiac Disease-Children's activities report (CD-Chart), in Israel and in Italy.

1.2. METHODS

The previously validated CD-Chart was administered in Italy to children aged 8-16, diagnosed with CD for over six months (n=40). Results were compared to an age and gender matched group of children with CD in Israel (n=40).

1.3. RESULTS

Preliminary results show that the CD-Chart's internal reliability was found acceptable for the entire group ($\alpha = 0.72$) and separately in each cultural group (Italy: $\alpha = 0.82$; Israel: $\alpha = 0.74$). A MANOVA analysis indicated significant differences between the two cultural groups ($F(6,73) = 11.38$, $p<.001$, partial effect size $\eta^2 = 0.48$). The following ANOVAs indicated significant cross-cultural differences in the number of activities in which the children participate, the frequency of participation or the need for preparation. The Italian children like participating in the activities significantly more than the Israeli children. However, the Italian children are significantly less involved in the preparation needed prior to participation in the various activities and showed significantly lower self-determination for such involvement than the Israeli children.

1.4. DISCUSSION

The CD-Chart presented cross-cultural similarities and differences in participation characteristics in various contexts of daily food-related activities. The CD-Chart can highlight important aspects of daily health management and direct clinicians to setting appropriate intervention goals to promote effective health self-management.

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Chapter 8 **– Current status and perspectives on the application of CRISPR/Cas9 gene-editing system to develop a low-gluten, non-transgenic wheat variety.**

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1.1. BACKGROUND

Common wheat (*Triticum aestivum*, 2n = 6x= 42, AABBDD) is a preferred staple food worldwide [1]. During 2018/19, the total global wheat consumption was 734.7 million metric tons, which increased by 759 million metric tons during 2021 [2]. However, in a huge number of individuals, the consumption of gluten (a storage protein of wheat) triggers several gluten-related disorders (GRD), including celiac disease (CD), which affects 1–2% of the world population [3]. CD is a T-cell mediated chronic enteropathy caused by the ingestion of immuno-dominant gluten peptides in genetically predisposed individuals who possess a specific human leukocyte antigen (HLA)-DQ2 and/or -DQ8 alleles [4,5,6]. Following a life-long strict gluten-free diet (GFD) is the only accepted treatment for CD [7]. Adherence to a strict GFD shows absolute regression in the celiac-associated symptoms (diarrhea, anemia, failure to thrive, weight loss, etc.) and is also suggested for other GRD [4,5,7,8]. Gluten is a ubiquitous

protein that is used universally not only in cereal-based products but also in numerous food and non-food industries [9,10]. Therefore, complete elimination of gluten from the diet is difficult [9,10]. Following a strict GFD also compromises the quality of life (QOL) of CD patients [11].

Gluten protein is primarily comprised of two classes of proteins, i.e., gliadins and glutenins. While gliadin makes dough viscous, glutenins provide a fine baking quality to wheat [12]. The existence of gliadins and glutenins as well as the balance of these two forms of proteins is critical for flour quality. Gliadin is further sub-divided into α-, γ-, and ω-subfractions, out of which α-gliadin primarily contains the critical epitopes that are responsible for CD development [13]. There are two fractions of glutenins, i.e., low and high molecular weight glutenins [12]. Gliadin is encoded by multiple gene families that are arrayed at Gli-2 loci on chromosome 6A, B, and D on specific loci in a repetitive sequence fashion [4,13,14,15,16]. α-gliadin contains a 33-mer peptide that is particularly rich in proline-glutamine sequences, and some of these α-gliadins are responsible for the development of CD. Human intestinal and pancreatic enzymes are unable to completely digest the complex amino acid sequence of α-gliadin, that is broken down into relatively larger peptides [4,17]. These peptides pass through intercellular junctions and enter in the lamina propria, where the tissue transglutaminase enzyme deamidates this gluten fraction. This modified fraction is recognized by the HLA-DQ heterodimers that are attached to antigen presentation cells. The HLA–gluten complex triggers T-cells to induce a pro-inflammatory cascade, which eventually leads to CD [17].

Wheat was introduced into the human diet about 10,000–12,000 years ago [18]. The first domesticated wheat varieties were diploid and tetraploid. Einkorn wheat only had one genome, i.e., the A genome (diploid). This wheat species was designated as *T. monococcum* and is rarely consumed by humans nowadays [18]. Tetraploid wheat was domesticated simultaneously with diploid wheat and contains two genomes (AA and BB); hence, it was termed tetraploid wheat. Durum wheat (*T. durum* or *T. turgidum*) is a tetraploid species of wheat that is mostly used to prepare pasta [18].

The currently most used bread wheat/common wheat (*T. aestivum*) is an allohexaploid species with three genomes (AA, BB, DD) resulting from natural hybridization between a tetraploid *T. turgidum* (dicoccum) carrying the AA, BB-genome, and the wild diploid species *T. tauschii* (DD-genome) [19]. While the introduction of the D-genome improved the bread-making properties of wheat, most of the immunogenic peptides in CD are encoded by the D-genome [18]. α-gliadin, which is encoded on D-genome, is more immunogenic and more easily recognized by the intestinal T-cells. Preliminary shreds of evidence suggest that primitive wheat (diploid or tetraploid) was safer and less immunogenic compared to currently used hexaploid wheat, as ancient wheat varieties had less immune-dominant protein fractions. However, this is strictly dependent on the particular genotype, not on the species [18,20]. Wheat varieties with low T-cell stimulatory epitopes may reduce the chances of developing CD. Exposure to an improved wheat variety with low-immunogenic wheat may not cause an intense immunological trigger to CD patients; hence. it could be useful for CD management [12,21].

Numerous efforts have been executed to develop a wheat variety with a lower percentage of immunological peptides (α-, ω-, and γ-gliadin), primarily by applying a combination of conventional mutation and breeding methods and RNA interference (RNAi) technology. However, a low-immunogenic wheat variety has not been able to be developed so far. [12,21,22,23,24].

In recent years, gene-editing techniques such as zinc finger nuclease (ZFN), and transcription activator-like effector nucleases (TALEN) have emerged as a promising approach to edit or delete the gluten fractions in wheat [25]. Another promising gene-editing tool, i.e., clustered regularly interspaced short palindromic repeats-associated nuclease 9 (CRISPR/Cas9) has evolved as a popular and novel second-generation genome-editing tool in science, medicine, and biotechnology. The CRISPR/Cas9 gene-editing system can remove or reduce the toxic fractions of gluten, resulting in a gluten-free or low-gluten wheat [13]. This gluten-free or low-gluten wheat would be a healthier choice for CD and GRD patients [12]. The use of hypoimmunogenic wheat flour in the preparation of gluten-free food or gluten-free products

may also be useful for reducing the increasing burden of gluten cross-contamination [26]. Due to genetic redundancy and genome complexity, wheat biology has straggled behind in adopting CRISPR/Cas9-based genome modifications. The key challenge now is to fully exploit the genome-editing ability of CRISPR/Cas9 to precisely alter gliadin genes, suppressing their immunogenic capability while maintaining their functionality and organoleptic properties.

So far, only a few studies have reported the application of CRISPR techniques to produce low-immunogenic/gluten-free wheat with novel agronomical traits. To the best of our knowledge, this review is among the first reports to provide an outline of the current status and contribution of CRISPR/Cas9 applications in the editing of the wheat genome. This article will help in bridging the research gaps that currently exist towards the development of wheat lines devoid of immunogenic gluten.

1.2. LITERATURE REVIEW

From January to April 2021, published literature related to the application of CRISPR to develop a low-immunogenic wheat variety was searched using the keywords <celiac and CRISPR>, <CRISPR in celiac disease>, and <Wheat engineering with CRISPR/Cas9>, <Low-immunogenic wheat and CRISPR> on electronic databases such as PubMed, Google Scholar, CrossRef, and CiteFactor. We also searched the references from the published articles that were found. No publication date was imposed. Only original articles published in the English language applying CRISPR/Cas9 for gene-editing in wheat crops were included. Review articles, protocols, scientific presentations, and Ph.D. theses were excluded; however, such articles were only used for reading purposes. Following these criteria, 68 studies were explored in total. Of them, 23 articles were found to be appropriate for the topic.

1.3. Genome-editing techniques: Tools that alter the genetic code

Genome editing or gene editing is an advanced technique that permits researchers to perform specific alterations in the genome of living cells. During 1970s, the development of genetic engineering (manipulation of DNA or RNA) opened up innovative possibilities in genome editing [27].

The main concept behind genome-editing techniques is to employ engineered endonucleases to create a site-specific DNA double-strand break (DSB), which is repaired either by non-homologous end joining (NHEJ) or by homologous recombination (HR) [12,25,28,29]. Genome-editing techniques have been categorized into two generations: (1) first-generation (i.e., mega-nucleases, ZFNs, TALEN) and (2) second-generation (e.g., CRISPR) gene-editing tools [25,30]. CRISPR is the latest gene-editing tool and is highly accurate, rapid, simple, and comparatively cheaper than other gene-editing tools [31,32]. The CRISPR/Cas9 system has been successfully applied for plant genome (Arabidopsis, rice, maize, and tomato) improvement and in various human diseases such as gastrointestinal, hematologic, viral, and cancer [13,33]. In a recent study, CRISPR/Cas9 significantly inhibited tumor cell growth as well as the migration and invasion of breast cancer cells [34].

1.4. CRISPR/CAS9: A new era of genome editing

The concept of CRISPR/Cas9 has been adopted from the defense machinery of bacteria [25,32,35]. When a virus (bacteriophage) attacks bacteria, the bacteria capture snippets of the genetic material of the virus and synthesizes DNA segments known as CRISPR arrays [25,32,35]. These CRISPR arrays memorize the virus, and on future invasions of the same or similar viruses, the bacteria then synthesize the RNA segments from the CRISPR arrays that target that virus. Bacteria use the Cas9 enzyme to cleave the targeted viral DNA sequence that eventually neutralizes the virus [36].

The CRISPR genome-editing system requires the design of guide RNA (gRNA) 20 nucleotides, which is complementary to the DNA stretch within the target gene. Along with the gRNA, the system also requires the Cas9 endonuclease, which together forms a ribonucleoprotein (RNP) complex that creates DSB in complementary DNA sequences [36,37]. In various human diseases, including neurodegenerative conditions, acquired immunodeficiency syndrome, and β-thalassemia, the CRISPR/Cas9 mechanism has been implemented effectively [13,33]. Recently, CRISPR/Cas9 has become a promising technique for trait improvement or functional genomics studies in various commercially relevant crops (*Oryza sativa*, *Zea mays*, *Solanum lycopersicum*, *S. tuberosum*, *Hordeum vulgare*, and *T. aestivum*). The use of the CRISPR/Cas9 system in plant genetic engineering is a relatively more contemporary and widely adopted tool for genome editing than ZFNs and TALENs [38,39]. The simplicity, multiplexed mutations, and robustness of CRISPR/Cas9 make it a preferred choice over first-generation genome-editing tools [40].

1.5. CRISPR/Cas9: The Machinery

The CRISPR/Cas9 system is present in diverse living organisms and fundamentally has a comparable core genetic organization [41,42]. They generally have multiple Cas genes encoding the Cas protein and several repeat DNA elements interspersed with short “spacer” sequences derived from foreign DNA. The AT-rich spacer sequence constitutes a code for the respective foreign genetic element that is used by the host prokaryotic to quickly identify any homologous sequence subsequently entering the host cells [43].

There are two main components of CRISPR: (1) single guide RNA (sgRNA), which is complementary to the target sequence, and (2) the Cas9 gene, which is adapted from Streptococcus pyogenes (SpCas9) and requires a G-rich (5′-NGG-3′) PAM (protospacer-adjacent motif) site that is responsible for generating DSB at a predesigned target DNA site [32,37,44]. sgRNA is a small sequence of nucleotides (18–21 nucleotides) that is complementary to the target DNA, and that has three PAM sites at the 3′ end followed by an

RNA scaffold [45]. The Cas9 protein comprises two functional domains: (1) the large recognition (REC) domain, which is the largest domain and is responsible for gRNA binding, and the (2) RuvC domain, which is a nuclease domain that cuts the single-stranded DNA. The NUC domain has two conserved endonuclease sites (RuvC and HNH) and a PAM interacting site. RuvC cleaves the non-complementary strand while HNH cleaves the complementary sequence of the sgRNA [12,36,45] (Figure 1).

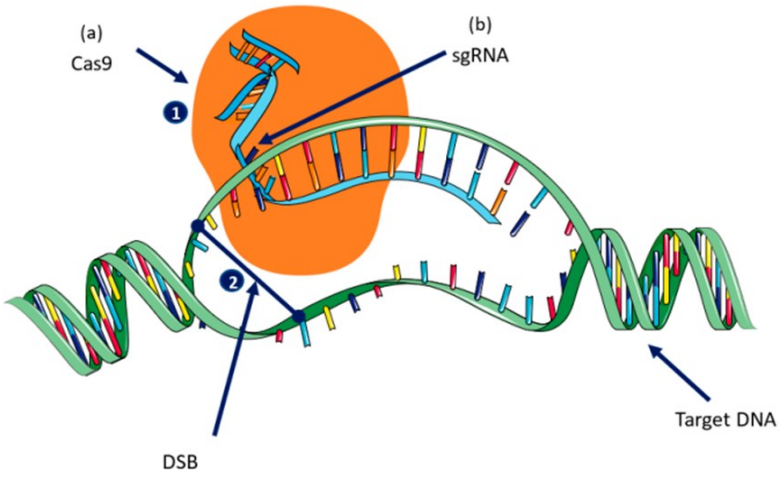

Figure 1. Mechanism of CRISPR/Cas9-mediated gene editing: there are two key components of CRISPR/Cas9: (**a**) Cas9 and (**b**) single guide RNA (sgRNA) **1:** The Cas9 nuclease is guided to its target DNA by the sgRNA. **2:** Cas9 causes a double-strand break (DSB) in the DNA that is repaired using either a non-homologus joining (NHEJ) or by homologus recombination (HR).

To neutralize foreign DNA in bacterial cells, the CRISPR/Cas9 system works in three stages [46,47]:

Stage I, acquisition stage: The invading DNA is recognized, and the spacer sequence is obtained from the target DNA. The repeated DNA sequence is inserted into the host CRISPR array to build an immunological memory [48,49].

Stage II, expression stage: The Cas9 protein is expressed at this stage, and the CRISPR array is transcribed into a precursor RNA transcript (pre-crRNA). The pre-crRNA and Cas9 protein are then hybridized by a non-coding trans-activating CRISPR-RNA (crRNA) and are processed into a mature RNA unit known as crRNA [50,51].

Stage III, interference stage: In the final stage, the mature crRNA directs the Cas9 protein to identify the DNA of interest, resulting in the cleavage and degradation of the invading foreign DNA [52,53].

The Cas9 endonuclease cleaves the DNA to generate blunt-ended DSB in the host genome, triggering a cellular DNA repair mechanism. The host DNA repair mechanism may either follow an NHEJ with small random insertion/deletion or by HDR, thus resulting in genome editing at the target locus [54]. In NHEJ, a highly error-prone repair mechanism, DSB, joins back together with the endogenous repair machinery, which generally introduces random insertions and deletions of the DNA. This could potentially lead to the disruption of the codon-reading frame and often results in gene knockout by forming a frameshift and premature stop codon. Alternatively, if a donor DNA template homologous to the sequence surrounding the DSB site remains available, the error-free HDR pathway is initiated, whereby precise deletions or insertions of the coding sequences can be achieved, leading to gene knock-in or deletion. The NHEJ leads to ablation gene mutation and can be used to generate a loss of function effect, whereas HDR can introduce precise changes in the genome by adding specific point mutations or by varying the length of the DNA segments [44,45].

1.6. CRISPR/Cas9: Challenges and Consequences in the Wheat Genome

The CRISPR/Cas9 system is a dominant gene-editing tool that has been successfully applied in more than 20 agronomically important crops species so far, and its application has led to yield improvements, disease resistance, biotic and abiotic stress, etc. [55]. In recent years, the CRISPR/Cas9 system has been employed in model plants such as Arabidopsis thaliana and Nicotiana benthamiana. Subsequently, this genome editing has been employed in major crops such as rice, wheat, maize, oilseeds, tomato, soybean, cotton, and potato [56]. Even though the CRISPR/Cas9 method has been validated in various crops, large-scale implementation in editing α-gliadins in wheat is still lacking. One of the major difficulties was the complex wheat genome. Hexaploid wheat *T. aestivum* (Bread Wheat) has a large genome

(approximately 17 Gbp) and has a high content of the repetitive sequences. This robust sequence prevents the insertion of target mutants in the genome and makes the editing process difficult [12]. Apart from this, modern wheat is an allohexaploid, i.e., it is the result of a series of naturally occurring hybridization events among *T. urartu* (A genome donor), T. speltoides (B genome donor), and T. tauschii (D genome donor) [57,58]. Due to the large and complex three homologous copies of genes (A, B, and D) in the genome, targeting multiple copies of a gene has always been challenging for gene-editing techniques [12,25].

However, due to the orthologues of the Cas9 gene, CRISPR/cas9 is now capable of targeting multiple genes simultaneously [13,59]. Currently, the CRISPR/Cas9 system is being used in the development of a low-immunogenic wheat variety. [12,31,60].

1.7. Application of CRISPR/Cas9 System in Wheat Genome Editing

In 2014, for the first time, the CRISPR/Cas9 system was used successfully in wheat protoplasts to edit the TaMLO gene (Mildew resistance locus O) [61]. The CRISPR TaMLO knockout lines have been successfully established to increase resistance against *Blumeria graminis f. sp. Tritici* (Btg), the causal organism of powdery mildew disease. The seventy-two T_0 lines obtained by biolistic particle transformation were analyzed for T7 endonuclease 1 (T7E1) restriction enzyme digestion, with four lines being reported to be edited for the T7E1 restriction enzyme site [62]. A T-DNA-based delivery system was commonly used to introduce sequence-specific nucleases (SSNs) and the gRNA. However, DNA–virus-based amplicons were used as an efficient construct delivery method and led to several-fold increases in terms of gene targeting efficiencies. The application of Geminivirus-based DNA replicons, such as a wheat dwarf virus (WDV) in wheat, resulted in a 12-fold increase in CRISPR/Cas9 expression compared to the ubiquitin reference gene, suggesting that it could be a future tool for genome engineering for complex genomes [63]. In another study, Kim et al., (2018) demonstrated gene editing in wheat protoplasts for dehydration-responsive element-binding protein 2 (TaDREB2) and ethylene-responsive factor 3 (TaERF3) using the wheat U6 snRNA promoter [60]. They

successfully transfected nearly 70% of protoplasts and confirmed the expressions of these edited genes with the T7 endonuclease assay. The two major pitfalls of CRISPR-mediated gene editing in crops (CMGE) were transgene integration and off-targeting into the genome. Off-target mutations were more common in crops with higher ploidy levels as well as in genes with a large number of paralogs. This shortcoming was overcome by using a biolistic delivery method for the CRISPR/Cas9 ribonucleoproteins (RNPs). However, RNP-based biolistic delivery offers a transient expression of CRISPR/cas9, and it also reduces the chances of off-target effects [31]. Later, in 2017, Liang et al. demonstrated the use of CRISPR/Cas9 RNP complex genome editing for grain morphometric traits such as grain length (GL), width (GW) genes TaGW2, and TaGASR7 in *T. aestivum*. This complex reduced off-target effects, as no off-targets were detected in the mutant *T. aestivum* population, and in addition, the complex became degraded in vivo. This DNA-free editing method had an advantage over traditional backcross breeding, which is a laborious and time-consuming procedure [64]. However, this method had some limitations, including low-efficiency rates compared to CRISPR/Cas9 DNA binary delivery systems. The RNP method is a more economical approach to achieve CRISPR/Cas9-based genome editing in perennial crop species if these limitations are overcome. Similarly, Wang W. et al., (2018) demonstrated the multiplexed gene editing of three wheat genes, TaGW2 (a negative regulator of grain traits), TaLpx-1 (lipoxygenase, which confers resistance to Fusarium graminearum), and TaMLO (loss of function, confers resistance to powdery mildew resistance), using the wheat U3 snRNA promoter [59]. Genome-editing efficiency was validated in wheat protoplasts, and the DNA was evaluated for mutations by next-generation sequencing (NGS) followed by Agrobacterium-mediated transformation and mutant screening. T0, T1, T2, and T3 were then subjected to statistical and phenotypic analysis, and three homeologous copies were observed for gene-editing efficiencies in wheat. In another study, the male sterility gene, i.e., Ms1 (male sterility 1) was targeted by CRISPR/Cas9 vectors, resulting in the generation of complete sterility in commercial wheat cv. Fielder and Gladius [65]. In 2018, Sánchez-León et al. used particle bombardment to demonstrate the potential of CRISPR/Cas9, this time with two gRNAs delivered separately.

They focused on genes that encode α-gliadins, seed storage proteins that have an epitope linked to CD. Twenty-one mutant lines in bread wheat and six in durum wheat were developed, both of which showed a significant reduction in α-gliadins and had up to 35 genes edited in a single line [12]. Howells et al. (2018) delivered gRNAs into wheat cells using Agrobacterium tumefaciens-mediated transformation, for example, to target the TaPDS gene, a gene that encodes phytoene desaturase [66]. Interestingly, Zhang et al. (2019) generated heritable targeted mutation in TaPinb, TaDA1, TaDA2, and TaNCED1. The combination of the Agrobacterium-mediated transformation process and the CRISPR/Cas9 gene-editing system greatly increased the mutagenesis efficiency in T0 generation. High editing frequency was observed in subsequent T1 and T2 generations. Since CRISPR/Cas9 activity is stable throughout generations, Agrobacterium-mediated transformation in wheat proved to be an ideal approach for genome editing [67].

Furthermore, Agrobacterium-mediated transformants contain only one or a few copies of the transgene, and transgene-free mutant lines are reasonably simple to acquire [68]. Kamiya et al. (2020) developed PCR-RFLP, a rapid method for detecting edited mutations in wheat that was validated by genomic clone sequencing. Three TaNP1 homoeo-alleles, which encode a putative glucose-methanol-choline oxidoreductase and that are needed for male sterility, were edited using the optimized CRISPR/Cas9 method. It was also demonstrated that having only one wild-type copy of each of the three TaNP1 genes was enough to maintain male fertility [69]. In a recent study, in order to reduce the expression of asparagine synthetase in grain without affecting its expression in any other part of the plant, Raffan et al. (2021) targeted the TaASN2 gene in *T. aestivum cv. Cadenza* using the CRISPR/Cas9 system. The study provided strong evidence that very low-asparagine commercial wheat varieties can be produced, allowing for the development of lower-acrylamide bread, cereals, biscuits, and other wheat-based foods [70].

The abovementioned studies successfully demonstrate that the CRISPR/Cas9 system has emerged as an effective tool to enable precise genome manipulation for the development of new wheat cultivars with improved novel traits. These studies have documented how

CRISPR/Cas9 has been successfully employed in the wheat genome to improve disease resistance, stress tolerance, increase yield, and nutritional improvement. We have summarized the twenty-three studies that used CRISPR/Cas9-mediated gene editing in wheat varieties in Table 1.

Table 1. Summary of functionally validated CRISPR/Cas9-based genome editing in wheat varieties.

S. No	Cultivar or Genotype	Target Gene (s)	Gene Function	Delivery Mode	SgRNA Promoter Used	Reference
1	*T. aestivum* cv. Cadenza	*TaASN2*	Genes encode for asparagine synthetase enzyme required in asparagine synthesis	Biolistic transformation	*Ubi-1*	Raffan et al., (2021) [70]
2	*T. aestivum* line H29 cv. Fielder & Ningchun4	*TaWaxy & TaMTL*	Pollen-specific phospholipase	*Agrobactrium tumefaciens* mediated transformation	*OsU6a*, *TaU3*, and *TaU6*	Liu et al., (2020) [71]
3	Wheat variety CB037	*TaNP-A1, TaNP-B, TaNP-D1*	Expression in the tapetum and required for male fertility	Biolistic and protoplast mediated transformation	*TaU6* and *TaU3*	Li et al., (2020) [72]
4	Common wheat (*T. aestivum* L.)	*TaQsd1, TraesCS4A02G110300 (IWGSC 2018)*	Control seed dormancy in wheat	Biolistic transient expression and *A. tumefaciens* mediated transformation	*TaU6*	Kamiya et al., (2020) [69]
		TaLOX2	Encodes for lipoxygenase 2; grain development and growth			
5	*T. aestivum* cv. Fielder	*TaABCC6 & TaNFXL1*	Susceptibility to Fusarium head blight (FHB)	Protoplast transformation	*TaU6*	Cui et al., (2019) [73]
		TansLTP9.4	FHB resistance			
6	*T. aestivum* cv. Fielder	*EPSPS*	The key enzyme involved in the metabolism of aromatic amino acid through the shikimate pathway	Protoplast transformation	*TaU6*	Arndell et al., (2019) [74]

Table 1. *Cont.*

S. No	Cultivar or Genotype	Target Gene (s)	Gene Function	Delivery Mode	SgRNA Promoter Used	Reference
7	*T. aestivum* cv. Fielder	*TaPinb*	Control grain hardness	*A. tumefaciens* (EHA105) mediated transformation	*TaU3*	Zhang et al., (2019) [67]
		TaDA1, *TaDA2*	Negative regulates seed and organ size			
		TaNCED1	Key enzyme in ABA biosynthesis pathway that confers resistance to drought stress			
8	*T. aestivum* cv. Fielder	*TaQsd1*	Control seed dormancy in wheat	*A. tumefaciens* (EHA101) mediated transformation	*OsU6*	Abe et al., (2019) [68]
9	*T. aestivum* cv. Kenong199 or Kenong9204	*TaALS*, *TaACCase*	The absence of the gene provides herbicide tolerance	Biolistic transformation	*TaU6*	Zhang et al., (2019) [75]
10	*T. aestivum* cv. Fielder & cv. Gladius	*TaMs1*	Encodes a GPI, which is required for pollen exine development	*A. tumefaciens* mediated transformation	*TaU6*	Okada et al., (2019) [65]
11	*T. aestivum* cv. Fielder	*TaCKX2-1*, *TaGLW7*, *TaGW2*, *TaGW8*	Wheat grain-regulatory genes	*A. tumefaciens* mediated transformation	*TaU6*	Zhang et al., (2019) [76]
12	*T. aestivum* cv. Fielder	*TaPin a* & *b*	Control grain hardness and contributes to anti-fungal properties	*A. tumefaciens* mediated transformation	*TaU6* & *TaU3*	Zhang et al., (2018) [44]
		TaWAXY or GBSS	Key enzyme in amylase biosynthesis			
		TaDA1	Negatively regulates seed and organ size by restricting the period of cell proliferation			
13	*T. aestivum* cv. Bobwhite	*TaGW2*	Negative regulator of grain weight, grain size enlargement, especially increased kernel width	Protoplast transformation	*TaU6*	Wang et al., (2018) [59]
		TaLpx-1	Encodes 9-lipoxygenase, silencing results in resistance to *Fusarium graminearum*			
		TaMLO	Knockout mutants provide resistance to powdery mildew			
14	*T. aestivum* cv. Chinese Spring	*TaPDS*	Reduction or loss of function results in a photobleaching phenotype	*A. tumefaciens* mediated transformation	*TaU6*	Howells et al., (2018) [66]
15	*T. aestivum* cv. Fielder or SBC0456D	*TaMs45*	Contribute to male fertility	*A. tumefaciens* mediated transformation	*TaU6*	Singh et al., (2018) [77]
16	Bread wheat, BW208 & THA53, & Durum wheat cv. Don Pedro	α-gliadin	Storage protein, adds to dough viscosity/plasticity and contains immunogenic epitopes for CD	Biolistic transformation	*TaU6*	Sánchez-León et al. (2018) [12]

Table 1. *Cont.*

S. No	Cultivar or Genotype	Target Gene (s)	Gene Function	Delivery Mode	SgRNA Promoter Used	Reference
17	*T. aestivum* cv. Chinese spring	*TaDREB2*	TF induced under water-deficient condition	Protoplast transformation	*TaU6*	Kim et al., (2018) [60]
		TaERF3	TF promotes tolerance under salt and drought stress			
18	*T. aestivum* cv. Bobwhite & AC Nanda	*TaLox2*	Encodes for lipoxygenase enzyme, which hydrolyzes linoleic acid, α-linolenic acid, and arachidonic acid	Neon transfection of protoplasts and microspores	*TaU6*	Bhowmik et al., (2018) [78]
19	*T. aestivum* cv. Bobwhite	*TaUbi, TaMLO*	Majorly responsible for powdery mildew vulnerability	WDV and Biolistic transformation	*TaU6*	Gil-Humanes et al., (2017) [63]
20	*T. aestivum* cv. Kenong 199	*TaGW2*-A1, -B1 & -D1	Negatively regulates grain weight and width	Biolistic transformation	*TaU6*	Liang et al., (2017) [31]
21	*T. aestivum* cv. Bobwhite & cv. Kenong199	*TaGASR7*	Gene controls the expression of grain length with pleiotropic effects on grain weight and yield	Biolistic transformation	*TaU6*	Zhang et al., (2016) [79]
		TaDEP1	Gene expression controls panicle size			
		TaLOX2	Encodes for lipoxygenase 2 and plays a critical role in grain storage and seed vigor			
		TaNAC2	TF promotes multiple abiotic stresses tolerance			
		TaPIN	Encodes for puroindoline gene and plays an important role in controlling the grain hardness			
		TaGW2	Negative regulator of grain weight, grain size enlargement, and especially increased kernel width			
22	*T. aestivum* L.	*TaMLO-A1, TaMLO-B1 & TaMLO-D1*	Loss of function confers resistance to Powdery mildew	Biolistic transformation	*TaU6*	Wang et al., (2014) [62]
23	*T. aestivum*	*TaINOX*	Biogenesis of plant cell wall	*A. tumefaciens* (GV3101) mediated transformation	TaU6 and CaMV35s	Upadhyay et al., (2013) [80]
		TaPDS	Involved in carotenoid biosynthesis that protects chlorophyll from photobleaching			

ABA, abscisic acid; TF, transcription factor; CD, celiac disease; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GPI, glycosylphosphatidylinositol; *OsU*, *O. sativa* small nucleolar RNA (snoRNA) promoters; *TaU*, *T. aestivum* snoRNA promoters; *Ubi-1*, *Z. mays* ubiquitin promoter.

1.8. RNA Interference (RNAi): Biology

The discovery of RNA-induced gene silencing provided a feasible alternate gene analysis technique through the simultaneous knockdown of the expression of multiple related gene copies. RNAi or RNA-silencing was discovered in Caenorhabditis elegans and plants during the late1990s as a post-transcriptional gene silencing (PTGS) mechanism that is able to target specific messenger RNA (mRNA) sequences and to downregulates protein expression [29,81,82,83]. RNA interference involves four main stages: (1) double-stranded RNA cleavage by the Dicer, (2) silencing complex (RISC) development, (3) silencing complex activation, and (4) mRNA degradation.

The first step in RNAi is the transmission of dsRNA into the cell, which is completely homologous to the target gene in sequence. The Dicer enzyme recognizes dsRNA and converts it into double-stranded short interfering RNA (siRNA) nucleotides of varying lengths in an ATP-dependent reaction, depending on the species. In the second step, the siRNAs produced by Dicer are integrated into the RNA-induced silencing complex (RISC), a multicomponent nuclease complex whose ability to conduct RNAi is inactive in this form [29,84]. In an ATP-dependent process, a helicase unwinds the siRNA duplex and further remodels the complex to form an effective RISC in the third step. The final step is to recognize and cleave mRNA that is complementary to the siRNA strand present in RISC. The target mRNA is cleaved into 22 nucleotide-long fragments, resulting in gene suppression or in the alteration of gene expression [85]. When cleavage comes to an end, the RISC leaves, and the siRNA is ready to be used in another mRNA recognition and cleavage period [86,87].

1.9. Role of RNAi in Modifying the Wheat Genome

Wheat RNAi has been successfully used to target a wide range of genes to date, but it has also been used to down-regulate protein encoded by multigene families, such as gliadins and glutenins [88,89]. In a short communication published by Gil-Humanes et al. in 2008, the authors used RNA interference to suppress the expression of particular γ-gliadins, demonstrating the feasibility of systematically silencing specific groups of gluten proteins. There were seven transgenic lines, all of which displayed decreased γ-gliadin content. The seven transgenic plants were fully fertile, and the grain morphology and seed weight were comparable to the wild-type grain morphologies and seed weights. The proportion of γ-gliadins was decreased by about 55–80% in the BW208 lines and by about 33–43% in the BW2003 lines as a result of this silencing [84]. In another influential study published in 2010, Gil-Humanes et al. down-regulated the gliadin expression (up to 63–93% for α-gliadin and 35–81% for ω-gliadin) in bread wheat by designing a set of hpRNAs containing a fragment of 361 bp that is widely conserved among α-, ω-, and γ- gliadins. There was a 1.5–2 log reduction in

the sum of the DQ2-α-II and DQ2-γ-VII epitopes and at least a 1 log reduction in the amount of DQ8-α-I and DQ8-γ-I epitopes in five of the transgenic lines. For three of the transgenic wheat lines, whole gluten extracts were unable to produce T-cell responses and had decreased responses for six transgenic lines [90]. Again in 2014, Gil-Humanes et al used flour from these transgenic wheat lines to develop a high-quality bread. The baking and sensory properties as well as the overall approval of the reduced-gliadin breads were comparable to those of regular flour but with up to 97% less gliadin content. Furthermore, low gliadin flour enhanced the nutritional properties because their lysine levels were considerably higher than that of regular wheat [91].

In a recent study, Haro et al. (2018) compared the digestibility of low-gliadin wheat (E82, low gliadin content, and reduced LMW glutenins) developed by the RNAi system from regular gluten-free bread in a subset of patients with no-celiac gluten sensitivity (NCGS). The findings indicated that eating low-gliadin E82 bread for one week was well accepted by NCGS patients, as the clinical effects were similar to those seen with gluten-free bread, and no variations in sensory parameters were observed. The data showed that the consumption of E82 bread does not cause adverse clinical symptoms, induces positive changes to the composition of the gut, increases butyrate-producing bacteria, and promotes the bacterial profile of the intestines, which plays a major role in gut permeability improvement in NCGS patients. However, this study did not address the relationship between the bacterial and fungal species of the gut microbiota. Further studies are needed to investigate bacterial and fungal microbiota modification in the gut upon the consumption of E82 bread [92].

These study findings indicate that RNAi is effective in reducing the levels of gliadins in wheat, which would be safer for gluten-intolerant consumers. However, it is still debatable if these wheat lines will become commercially viable or whether the discoveries will be converted into something of economic utility.

1.10. Applications of CRISPR/Cas9 and RNAi: A Comparative Analysis

Gene modifications are powerful tools that have been widely used in past decades to understand fundamental biological processes of interest and their function. RNAi has previously been the major dominating genetic tool for manipulating genes and performing genetic function studies in various areas of crop development. However, the rapid growth and use of CRISPR/Cas9 have been successfully applied in many agronomic crops. Both RNAi and CRISPR/Cas9 are useful tools for modifying genomic DNA and changing genetic information, including gain-of-function and loss-of-function. CRISPR/Cas9 and RNAi are widely explored from a technical and methodological standpoint (Figure 2).

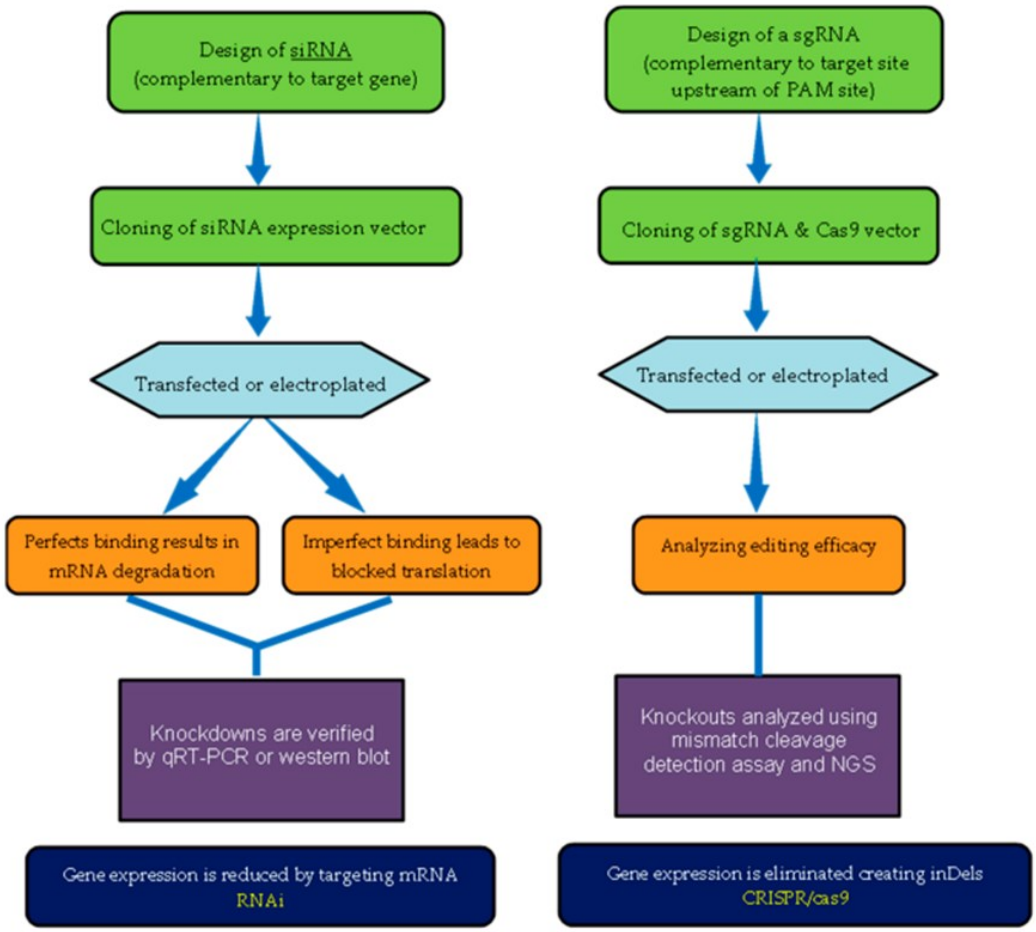

Figure 2. Schematic representation of RNAi and CRISPR/Cas9 experimental workflow. Abbreviations: NGS, next-generation sequencing; inDel, insertion–deletion mutation.

A comparison of the scope of CRISPR/Cas9 and RNAi in research and practical studies is discussed below.

Knockout vs. Knockdown: CRISPR causes gene knockouts, which occur when DSB is made within the coding region of the gene [93]. This DSB triggers NHEJ or HDR [94]. RNAi reduces or knocks down gene expression at the post-transcriptional level by targeting RNA, where it generates a hypomorphic phenotype in contrast to the true null knockout that is possible with CRISPR/Cas9.

Ease of Design: The designing of a siRNA requires the sequence information of the corresponding mRNA transcript. siRNA is designed to target any transcript at almost any locus, but its activity is influenced by other factors such as the structure of the mRNA target region, base preferences, and overall siRNA G/C content. The design of a siRNA is a critical component of an effective RNAi experiment. CRISPR, on the other hand, requires information about the genomic DNA sequence. A CRISPR system such as CRISPR/Cas9 requires the protospacer adjacent motif (or PAM), a short DNA sequence required to cleave the targeted DNA. Depending on the type of Cas9, the PAM sequence recognizes the 5′-NGG-3′ site (where “N” can be any nucleotide base) [95].

Timespan: The mode of action differs between CRISPR/Cas9 and RNAi, which greatly impacts the duration of gene expression. siRNA knockdown exhibits significant gene repression within only 24 h of treatment. However, genome editing with CRISPR/Cas9 may result in a permanent effect, which usually requires the selection of cells with the desired InDels (insertion-deletion mutation) in all alleles, a time-consuming process depending on the specific need [96].

Flexibility: Targeted gene editing, especially CRISPR/Cas9, is heritable, i.e., once it introduces the change in the genome of the host cells, its physiological effect is passed on to the next generation. RNAi, unlike CRISPR/Cas9, does not result in a stable gene fragment, mutation, or inactive gene [97]. The in vivo application of RNAi is limited to instances where gene expression is suppressed post-transcriptionally.

Off targets: Since the discovery of RNAi, off-targets are one of its biggest limitations. siRNA induces the silencing of non-target mRNA with a limited sequence complementarity, via interaction with 3′UTR. However, it has been discovered that a single siRNA could potentially repress hundreds of transcripts with limited complementarity. However, the CRISPR/Cas9 system also has some sequence-specific target effects that can be overcome over a short period of time. This shortcoming was rectified through the use of the Cas9-nickase, a mutation in one of the Cas9 nucleases that reduces off targeting by 50-1500 fold [98]. While optimal siRNA design and chemical modifications have reduced the off-target activity of RNAi, a recent comparative study found that CRISPR/Cas9 is less susceptible to off-target effects than RNAi [99].

1.11. CRISPR/Cas9 Is a Method-of-Choice for Wheat Genome Editing

The recent emergence of multiple technologies for modifying gene structure has reformed agriculture and has resulted in improved that were not possible with conventional breeding procedures alone. These genetically modified crops have created huge economic and environmental benefits and are widely accepted across the world. Over the past decade, the RNAi technique has been widely used in both dicotyledon and monocotyledon to improve plant growth and productivity, impart resistance against pathogens, and create tolerance against various biotic stresses. RNAi or post-transcriptional gene silencing (PTGS) is a cellular mechanism conserved in most eukaryotic organisms that leads to the loss of functionality of a gene by blocking the messenger RNA (mRNA) molecules needed for protein formation.

Since RNA expression constructs are typically delivered as transgenes, through plant transformation, or as part of virus vectors, they must go through genetically modified organism (GMO) regulatory procedures to gain commercial approval. Several other techniques for stable genetic modifications, collectively known as gene-editing techniques, have been developed in parallel to the production of RNAi [100]. CRISPR is one such novel second-generation genome-editing system that has been exploited to generate desired mutations,

facilitating the development of crops with any given desirable trait. In the last decade, due to its simplicity, speed, and efficiency, CRISPR/Cas9 has quickly become a standard technique for modifying endogenous genes in almost all crop species. The CRISPR/Cas9 system has target specificity, as the target sites are recognized by the Watson and Crick model, and the off-target sites are identified through sequence analysis [101]. CRISPR/Cas9 represents significant technical advances for genetic engineering, but attempts must be taken to increase its productivity in a variety of plant species with large, complex genomes.

While the utility of the CRISPR/Cas9 has been studied in many diploid plants, its applicability in polyploidy crops with complex genomes (wheat) is still a challenge. Wheat is an allohexaploid that consists of three sets of closely associated homogeneous genomes [37,59]. Therefore, simultaneously targeting three or even more copies of a gene is a problem for editing wheat genomes, and attempting to knock out any of a gene's copies does not result in phenotypic modifications due to genome buffering. Wheat, on the other hand, which has a large genome and a high content of repetitive DNA (80–90%), makes it unusually recalcitrant to introduce targeted mutations. However, due to the availability of new orthologs of the Cas9 gene, sgRNA design in the CRISPR/Cas9 system can be effectively programmed to target several genes.

Another concern is that there are only a few wheat varieties that can be easily transformed, which restricts the use of CRISPR in wheat. However, there are well-established protocols for the transformation of the CRISPR/Cas9 construct using Agrobacterium-mediated and bombardment or biolistics delivery methods [35]. In addition, using recently designed CRISPR-based multiplex genome-editing toolkits, it is possible to accomplish simultaneous multiplex targeted modifications by co-transforming multiple sgRNAs. Evidence from published data shows that the CRISPR/Cas9 technique has been successfully applied to numerous wheat varieties to engineer novel agronomic traits associated with yield, quality, and resistance to biotic and abiotic stresses, etc. CRISPR/Cas9 is highly desirable for achieving the goal of editing α-gliadin genes in the development of wheat lines with fewer gluten genes and/or gluten genes with inactivated CD epitopes in bread wheat [13].

1.12. DISCUSSION

Celiac disease is a complex disorder in which the function of a major non-genetic factor, i.e. 'gluten' has been well established. A life-long GFD is the sole cure for CD [7]. However, a GFD, on the other hand, is difficult to follow because gluten is a commonly used food additive that can be found in items that do not initially contain gluten [10,102]. Furthermore, gluten-free products can be less healthy nutritionally since they are made with high levels of fat and sugar to create a texture that resembles the normal and unusual viscoelastic properties of wheat. Additionally, studies have linked GFD to the lower consumption of dietary fiber, and some commercially available GFPs have lower vitamin B, folate, and iron content [103]. Moreover, the exclusion of gluten from the diet of CD patients reduces their QOL [11]. Rigorous efforts have been conducted to explore an alternative treatment that allows CD patients to consume wheat [17]. The use of a special wheat variety devoid of T-cell stimulatory epitopes may be a viable and successful alternative option. Currently, the only safe alternative would be the development of a "low-gluten/gluten-free" wheat variety that does not contain toxic peptides while retaining the basic properties of wheat [17,104,105].

Since bread wheat has a complicated hexaploid genome, the successful breeding of this crop is heavily reliant on the understanding of functional genomics. Advanced crop functional genomics, which can show how wheat genetics determine function, must now be complemented with existing modern breeding efforts. Plant biologists, based on their understanding of functional genomics, can alter the structures and functions of selected key genes through "genetic manipulation" based on their understanding of functional genomics. RNAi and CRISPR/Cas9 are two advanced technologies that can be used to modify or remove CD inducing epitopes from wheat gluten. The RNA silencing technique shows favorable results in this regard. Several research groups have explored the possibilities of using RNAi in silencing the toxic fragments of gliadin and have found promising results [84,90,106]. In a fundamental study, Gil-Humanes et al. used RNA interference to reduce gliadin gene expression by 97%, therefore preventing the stimulation of T cells from CD patients without

compromising seed germination or dough quality [90]. The RNAi wheat line (E82) developed by Javier Gil-Humanes and colleagues was exceptional because of its low ability to produce an immunogenic response and its ability to retain its organoleptic and agricultural properties. The study was conducted in volunteer NCGS patients and was compared with a GFD to test the acceptability, digestibility, and safety of the bread made from the wheat flour of the E82 line with all of the gliadins being strongly downregulated. Furthermore, in non-celiac wheat sensitivity patients, eating bread made with this low-gliadin line encourages a stronger gut microbiota profile than gluten-free bread [91].

Since the transgenic RNAi construct persists in the wheat genome to silence the genes, these plants are subjected to GM control, which is costly, time-consuming, and unpredictable in the European Union (EU) [21,26,107]. Unlike other breeding methods, the implementation of genetic transformation is strongly regulated in the EU. This contradicts the fact that the cultivation of GMOs is essentially prohibited in the EU, but importation is permitted [108]. As a result of this stringent regulation, the general population is concerned about GMOs on a variety of levels, including their environmental impact and whether GM foods pose any health risks.

Emerging targeted genome-editing technologies offer plant breeders a new and effective tool. In terms of genome editing, SSNs have been used to alter the target position of genes present in the genome. SSN, similar to CRISPR/Cas9, causes DSB, which can be repaired using an NHEJ or HR [54,109]. Unlike transgenic modifications, which require the insertion of foreign DNA sequences into a genome, gene editing may produce genetic variation through precise and direct changes in the genes of interest without integrating foreign DNAs or, if so, null segregants containing no recombinant DNA but that maintain the desired mutations and that can be easily retrieved. Instead of being categorized as GMOs, such edited plants could be considered non-transgenic plants. Moreover, it is expected that the Court of Justice of the European Union (ECJ) will exempt CRISPR/cas9 modified crops from the existing European law that has limited the planting and sale of GM crops [110].

In plants, CRISPR/Cas9 has already been shown to be a very highly efficient genome-editing system [111,112,113]. The hexaploid genome and large genome size are the major obstacles to CRISPR use in wheat biology. However, because of the high efficiency of CRISPR/Cas9 it is possible to acquire mutations in multiple genomes in a single polyploid plant. Finally, multiplexed genome editing using the CRISPR/Cas9 library can be easily accomplished using the monomeric Cas9 protein and a variety of sequence-specific gRNAs [25,59]. Moreover, genome editing through CRISPR/Cas9 entails a few simple steps that enable smaller laboratories with basic plant transformation abilities to perform genome editing in crop plants. The ease of use of CRISPR/Cas9 programming and its potential for multiplexed target identification have fueled the success of this low-cost and easy-to-use technology. According to some research, while CRISPR/Cas9 can cleave a target site, it can also cleave sites that do not match the target site [61]. In gene therapy, this off-target effect is a major problem, but it may not be a concern in plant biotechnology. Back-crossing or crossing with wild-type plants could be used to remove the putative off-target mutations. Furthermore, the use of web-based software to develop target sites is advised in order to mitigate off-target mutations by exploiting computation.

Susana Sánchez-León et al. utilized CRISPR/Cas9 genome editing technology to reduce the number of α-gliadins in the seed kernel precisely and effectively, resulting in gluten-free bread and durum wheat lines [12]. Interestingly, the bread wheat line (plant 10) had the highest decline in α-gliadins (82%) and γ-gliadins (92%) as well as the highest overall gliadin reduction (82%). Amongst the durum wheat lines, plant 2 had the highest overall gliadin reduction (69%). By improvising the current intricacies in the methodology, it is possible to develop a safe variety of wheat for CD patients. If such gluten-free wheat maintains its natural taste, it would be easier for CD patients to adhere a completely to the GFD. About 50% of CD patients do not follow a strict GFD for multiple reasons, including the unavailability of gluten-free food and the appalling palatability of the GFD, etc. [8,114]. A safe wheat variety for CD patients would be helpful to eradicate this problem, and CRISPR/Cas9 technology has the potential to produce such a variety of wheat [26]. However, CRISPR-modified wheat flour may lead to

problems such as dough formation that need to be resolved. Nonetheless, multiple studies support the fact that CRISPR/Cas9-mediated gene editing has overcome the current wheat genome complexity for genetic improvement (Table 1). The use of CRISPR/Cas9 for gene knockout and the Cas9 system for the expression regulation of any gene of interest would aid in the development of non-transgenic wheat plants. CRISPR technology is evolving, and existing systems are being engineered to include innovative capabilities. Moreover, exciting new CRISPR systems with novel functions are also being discovered.

1.13. CONCLUSIONS

The CRISPR/Cas9 gene-editing system is capable of editing the complex hexaploid wheat genome (T. aestivum). The availability of whole-genome sequence information for wheat along with the advancements in the CRISPR/Cas9 technique could provide possibilities for the development of a “hypo-immunogenic-wheat variety”. CRISPR/Ca9 could be a breakthrough for providing a promising dietary treatment for celiac disease. However, until now, only a limited number of studies have applied the CRISPR/Cas9 system to develop low-gluten wheat. Further studies are required to apply the CRISPR/Cas9 gene-editing system efficiently for the development of a celiac-safe wheat variety and to establish it as a “tool to celiac safe wheat”.

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Chapter 9 **– CONCLUSIONS**

The only available treatment of CD is a lifelong adherence to the GFD. A strict GFD is, often, difficult to maintain, owing to small levels of gluten contamination in food products, high costs, restricted availability of gluten-free food alternatives, low palatability, and cultural practices, leading to a substantial social burden. In past decade, researchers have become increasingly interested in CD prevention, and research has been directed to understand the role of environmental factors such as the amount and type of ATI in grains as potential triggers of disease. The results of our study demonstrated that both wheat genotype (modern versus ancient grains) and phenotype (different production area) can influence the immune response, and a significant correlation was found between the amount of ATI and their biological activity, so that the varieties with lower amounts of ATI induced a lower production of pro-inflammatory cytokines. The identification of wheat varieties with absent or low toxicity may have important implications for patients in terms of greater palatability, compliance to treatment, nutritional quality and quality of life. These results may also have profound implications on the economic burden of CD. In Italy, it has been estimated that approximately 500000 individuals are affected with CD and the costs of the GFD are supplied by the public health service. The prevention of the disease and the availability of alternative therapies to GFD may significantly reduce the impact on the National Health System. Further studies are needed to identify new possibilities for the production of cereals with reduced ATI content/biological activity and evaluate their effectiveness in clinical trials.

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LIST OF AUTHOR'S PUBLICATIONS

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