CELIAC DISEASE

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CELIAC DISEASE

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EDITORIAL
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Editorial: Celiac Disease

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Editorial on the Research Topic

Celiac Disease

The manifestations and broad impact of Celiac disease (CD), a chronic inflammatory disorder of the small bowel mediated by immune responses to triggering peptides from dietary gluten, are now increasingly recognized (1). This special issue draws together a series of reports focusing on different aspects of this important pediatric condition.

EPIDEMIOLOGY OF CELIAC DISEASE

The presentation patterns of CD have been observed to have changed and evolved in recent decades. Popp and Maki outline the key changes in the epidemiology of CD and illustrate the likely environmental and other factors that may have derived these changes.

PATHOGENESIS

In recent years, there have further advances in our understanding of CD. These include the role of cereals, intra-luminal digestion, epithelial barrier function, tissue transglutaminase enzymatic activity, genetic factors, and immune responses.

In their review, Tye-Din et al. delineate the important aspects of the aetiopathogenesis of CD. Although the emphasis is upon genetic susceptibility and dietary exposure to cereal peptides, this report draws attention to other potential factors including host-pathogen interactions and concurrent environmental factors. As outlined, further advances in understanding these key aspects should assist in advances in diagnosis and management. The authors further emphasize that additional work is required to understand environmental triggers, interactions with the microbiota and raise potential for prevention and enhanced management and even prevention.

Smigoc Schweiger et al. explored the relationship between polymorphisms in genes linked to the inflammasome in CD and in type 1 Diabetes. The researchers evaluated the presence of polymorphisms in selected genes in small groups of Slovenian children with T1D, CD or both conditions. Although they documented a link between the development of T1D and a polymorphism in the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene, this polymorphism was associated with protection from CD. While the mechanism of this protective benefit was not elucidated, this work emphasizes the importance of non-HLA genes in the development of CD. Further attention to this enzyme and it's potential adverse impacts is required.

In another aspect of the pathogenesis of CD, Torsten and Aaron review the potential impact of microbial transglutaminase (TG). While it is well-established that mucosal TG plays an important role in de-amidating cereal peptides, the role of microbial sourced TG has not been fully considered. The authors outline the chemical nature of this enzyme, outline the current industrial roles, and characterize how this enzyme could also impact upon CD.

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MANIFESTATIONS OF CD

Although CD in primarily a condition affecting the small bowel, there may be various and widespread manifestations outside the gut. Nardecchia et al. reviewed the patterns of these extra-intestinal manifestations (EIM) with emphasis upon the particular patterns of these features in children (as distinct to adults). The authors also explored the potential mechanisms for CD-related EIM. Two key mechanisms were detailed: those consequent to intestinal malabsorption and those consequent to immune responses. This work helps to emphasis the nature of these EIM and draws attention to the early recognition of these features in children who may not present with any gastrointestinal symptoms.

PATIENT MANAGEMENT

Meijer et al. discuss the potential for prevention of CD. Recent work has shown that early feeding steps may not prevent CD in children at greater genetic risk (2–4) and further work is required in this population. Further, primary, secondary, and tertiary prevention and management strategies are outlined and emphasized. At present, such strategies are not fully understood, and further work is required to answer the outstanding questions. This may enhance outcomes in due course.

In addition, Yoosuf and Makharia highlight the range of novel therapies that are being evaluated and considered as options for the management of CD. None of these therapies are established as valid and effective options, and it is not clear if any of these will be complete alternatives to the GFD. But none-the-less, these options provide optimism for the future.

CONTROVERSIES

There are several controversies in the management of CD. Cohen et al. (5) recently reviewed the issue of how much dietary gluten is safe in people with CD. Total daily gluten consumption that seems to be safe for most CD patients is <50 mg gluten, nevertheless, some CD patients need as little as 10 mg of gluten daily to promote development of intestinal mucosal abnormalities. Therefore, they recommend that until new data will be available the recommendation for children should be a GFD with <20 ppm as defined by the Codex.

Two articles explore key controversies in the management of a GFD for CD.

Spector Cohen et al. reviewed the dilemma of including oats in a GFD. It appears that pure oats are well-tolerated by most CD patients at moderate amounts (20–25 gr/day dry rolled oats for children; 50–70 gr/day for adults). Nevertheless, due to existing uncertainty it is suggested that oats should be added with caution to a GFD, only after all CD symptoms including weight loss and growth disturbances have resolved, after at least 6 months of a conventional GFD and only after normalization of serology. Furthermore, these patients should be closely monitored.

While the GFD is currently the only intervention available for individuals with CD, Lerner et al. outline the potential adverse implications of a GFD. This article highlights that a GFD could lead to nutritional deficiencies and mental health outcomes, and emphasizes the importance of dietetic oversight of a GFD.

CONCLUSIONS

Together the articles comprising this special issue provide important and timely updates about the current status of CD in children across the world. Each report raises questions and indicates aspects that require further attention and scientific enquiry. While the understanding of the pathogenesis and manifestations of CD has advanced greatly in recent years, there remains controversy and lack of a cure.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Celiac Disease: A Review of Current Concepts in Pathogenesis, Prevention, and Novel Therapies

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Our understanding of celiac disease and how it develops has evolved significantly over the last half century. Although traditionally viewed as a pediatric illness characterized by malabsorption, it is now better seen as an immune illness with systemic manifestations affecting all ages. Population studies reveal this global disease is common and, in many countries, increasing in prevalence. These studies underscore the importance of specific HLA susceptibility genes and gluten consumption in disease development and suggest that other genetic and environmental factors could also play a role. The emerging data on viral and bacterial microbe-host interactions and their alterations in celiac disease provides a plausible mechanism linking environmental risk and disease development. Although the inflammatory lesion of celiac disease is complex, the strong HLA association highlights a central role for pathogenic T cells responding to select gluten peptides that have now been defined for the most common genetic form of celiac disease. What remains less understood is how loss of tolerance to gluten occurs. New insights into celiac disease are now providing opportunities to intervene in its development, course, diagnosis, and treatment.

Keywords: celiac disease, gluten, T cells, microbiome, pathogenesis

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INTRODUCTION

Celiac disease (CeD) is a chronic immune-mediated enteropathy precipitated by to dietary gluten in genetically predisposed individuals (1). Current diagnosis is based on demonstrating the enteropathy in small intestinal biopsies where histologic examination shows villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis, and the presence of circulating CeD-specific antibodies to tissue transglutaminase (tTG), deamidated gliadin peptides (DGP), and endomysium (EMA). In children who have symptoms suggestive of CeD, a strongly positive tTG antibody (tTGA) titre and a CeD-associated HLA genotype, the diagnosis of CeD may be possible without the need for small intestinal biopsy (2). Since the 1950s when gluten was identified as the causative trigger of CeD, a strict and lifelong gluten-free diet (GFD) has been the mainstay of treatment.

While CeD is common around the globe and is rising in prevalence in many populations, it is frequently undetected in clinical practice (3). Symptomatic and untreated disease is associated with elevated morbidity and mortality and impaired quality of life (4–7). The clinical presentation is broad and includes gastrointestinal upset, chronic fatigue, nutrient deficiencies, poor growth, and failure to thrive. Extra-intestinal manifestations are common, and while they were once considered more frequent in adults than children with CeD, recent data suggests the frequency is similar in CeD children, although the types and rates of recovery differ (8). In children, extra-intestinal manifestations include short stature, anemia, delayed puberty, dental enamel hypoplasia, reduced bone density, oral ulcers, liver and biliary disease, and dermatitis herpetiformis. Poor growth and anemia tend to be the most common and there is a correlation with more severe histological damage at diagnosis compared to children with a gastrointestinal presentation or screen-detected cases (9). Insidious effects of undiagnosed CeD in children include behavioral disturbances and reduced educational performance (10).

CeD is also associated with an increased risk of autoimmune diseases including Hashimoto's thyroiditis, Graves' disease and type 1 diabetes (T1D) (11–14). A large Danish population study revealed the prevalence of autoimmune diseases was 16.4% among CeD patients compared with 5.3% in the general population in 2016 (12). Approximately 5% of patients with CeD have T1D and ∼6% of T1D patients have CeD (15). In Northern Sardinia, a population with a high prevalence of CeD (16), patients with autoimmune thyroiditis had a 4-fold greater prevalence of CeD than the general population and while iron deficiency was present in almost half, none had gastrointestinal symptoms (17). The co-occurrence of autoimmune diseases supports the concept of shared genetic and immune pathways contributing to immune dysregulation and loss of self-tolerance, however it remains unclear whether CeD directly leads to other autoimmune disease and whether early diagnosis and treatment with a GFD alters this risk (18). Next generation sequencing of the HLA region shows that extended class II haplotypes differ between populations (19); this may partially explain regional differences in the degree of association between CeD and autoimmune disease. The strong association of CeD with autoimmunity, especially T1D and autoimmune thyroid disease, supports screening for these conditions in CeD patients.

The consumption of gluten containing foods is necessary for CeD to develop. Gluten is the viscoelastic protein that remains after washing dough and is composed of alcohol-soluble gliadins and alcohol-insoluble glutenins. The rheological properties of gluten allow it to impart a light and extensible texture to food making it highly favored in the food industry. Modern wheat gluten arises from a hexaploid genome, making it heterogeneous and more genetically complex than the human genome. Similar proteins rich in glutamine and proline (thus, the collective term prolamins) are found in barley and rye and termed hordeins and secalins, respectively, and are also toxic in CeD. Avenin, the prolamin component in oats, is phylogenetically distinct from wheat, barley and rye prolamin. Oats are considered safe for consumption by most people with CeD, although adverse immune and clinical effects have been reported (20–22) and some cultivars may be more immunogenic (23). Several expert reviews have concluded that further research into the toxicity of oats in CeD is needed (24, 25).

Why CeD develops in some people remains unanswered. Epidemiologic and prospective observational studies implicate a range of environmental factors that impact disease development. A role for the microbiome in the development of tolerance and disease pathogenesis is also emerging. Genetic and immunological studies have revealed the importance of key HLA and non-HLA susceptibility genes in disease development and a long-lived pathogenic population of gluten-specific T cells targeting certain gluten peptides (T cell epitopes). The emerging story of CeD development is one where environmental factors increase the risk of CeD in genetically predisposed individuals by shaping the immunologic context in which gluten is presented and shifting the balance from gluten tolerance to reactivity, and that this may be in part mediated through microbiome-host interactions. Contemporary clinical issues of importance include expediting the detection and diagnosis of CeD, improving and quality of life and health outcomes for those diagnosed, and developing treatments that are more effective and less burdensome than the current approach of a lifelong and strict GFD. This article will review current knowledge about CeD development and pathogenesis and how this knowledge is being applied to explore novel diagnostic, treatment and preventative approaches.

THE ROLE OF ENVIRONMENT IN DISEASE DEVELOPMENT

CeD is a global disease that has been reported in Western and Eastern Europe, North America, South America, Asia, Oceania, and Africa (3, 26). It appears to be relatively uncommon in southeast Asia and sub-Saharan Africa. Recent reports from China suggest the illness could be substantially unrecognized there however more biopsy-based studies are required (27). In a systematic review and meta-analysis, the global seroprevalence and biopsy-confirmed prevalence of CeD was estimated to be 1.4% and 0.7%, respectively (3). The sero-prevalence of CeD in the US from National Health and Nutrition Examination Surveys (NHANES) was 0.7% and, consistent with a range of population studies from around the globe, showed that most cases remain undiagnosed in the community (28). Since CeD is frequently undiagnosed an active case-finding approach is considered best practice.

The prevalence of CeD varies with sex, age, and geographic location with the frequency of predisposing HLA haplotypes in the general population and per-capita wheat consumption the two major determinants of prevalence. There is a modest gender bias in favor of females (29). Familial clustering in CeD is common with 10% of first-degree relatives of a CeD sufferer affected. The high concordance rate for monozygotic twins (∼80%) compared to HLA-identical siblings (∼30%) and dizygotic twins (∼10%) underscores the importance of both

genetic factors (HLA and non-HLA genes) and the environment in CeD risk (30).

The high prevalence of CeD observed among populations living in areas with a high consumption of wheat products are highly suggestive for involvement of dietary gluten in CeD development (26). Although intake of gluten is necessary for CeD to develop, it does not solely explain why not all genetically-predisposed individuals consuming gluten develop CeD and why the disease can develop later in life despite many decades of gluten intake. Significant differences in the prevalence of CeD between people of similar genetic background and wheat intake living in nearby regions (for example, Finland and Russian Karelia) is strong evidence that the risk of CeD is influenced by other factors apart from genetic susceptibility and wheat consumption (31). Indeed, population studies have implicated a range of environmental factors associated with CeD risk (summarized in **Table 1**). Heterogeneity of study design has yielded conflicting results in the search for triggers in CeD. We have limited our review to focus on the association between CeD and diet, infections, antibiotic use, and delivery mode.

Infant Feeding

The steep rise in CeD incidence in young children after changes in the Swedish national infant feeding recommendation in the mid-1980s that suggested postponing gluten introduction from 4 to 6 months of age hinted that timing of gluten intake influenced CeD risk (61). However, the epidemic of CeD that occurred in Sweden occurred simultaneously with companies raising the gluten content in commercial baby formulas and was confounded by an observed protective effect of long breastfeeding duration (62). This made it difficult to disentangle whether timing or amount of gluten intake in relation to weaning impacted on the risk of CeD. The hypothesis that timing of first gluten exposure was associated with CeD was further supported in a study that found infants exposed to gluten either early (<4 months) or late (>7 months) were at an increased risk (63). Since this first prospective study was published several follow-up papers from larger longitudinal prospective birth cohorts summarized in two recent systemic reviews with meta-analysis (32, 33) have not been able to confirm the previous findings that either age of gluten introduction or breast-feeding influence CeD risk.

Although there are wide differences in gluten intake between countries (64), it is not entirely clear whether the quantity of gluten intake during early childhood affects the risk of CeD. A Swedish retrospective case-control study indicated that children that later developed CeD consumed larger amounts of gluten before the age of 2 years than healthy children (62). This finding was in line with another cross-sectional study from the same group that observed a lower prevalence of CeD in a birth cohort reporting a lower gluten consumption in children born *after* (65) as compared to children born *during* the years of the Swedish epidemic (66). In a nested case-control study, a high intake of gluten amount increased the risk for CeD in Swedish children (35). However, whether gluten intake contributes to CeD development is still controversial as another multicenter study consisting of five other European countries found no association with CeD and gluten amount except for children carrying the lower-risk HLA-DQ2.2/DQ7 haplotype (67). Larger prospective studies with a longer follow-up are underway and will shed light on whether gluten intake is an independent risk factor in CeD.

Infections

Several studies have shown that children that later develop CeD are more frequently affected by infections during early life (37, 68, 69). One limitation is that these studies are based on questionnaires filled in by parents and the type and site of infection is not specified. In a multicenter, prospective birth cohort study parents that reported a gastrointestinal infection 3 months prior to seroconversion of tTGA were at an increased risk of CeD autoimmunity later in life (38). There is also an effect of seasonality on the risk of developing CeD, hypothesized to be caused by viral infections occurring during a vulnerable period of immune development. This is supported by the association with frequent rotavirus infections and increased risk of CeD autoimmunity from longitudinal prospective studies (39) and a protective effect of rotavirus vaccination (38).

How infections trigger CeD development remains unexplained. Gastrointestinal infections may increase gastrointestinal permeability to increase the passage of gluten across the mucosa, or elevate tTG expression that can increase the generation of immunogenic gluten peptides. Molecular mimicry could possibly occur if the foreign antigen (such as a virus or bacteria) shares sequence or structural similarities with gluten itself and then initiates an anti-gluten response. Several studies have shown antibodies to adenovirus (70–72) and rotavirus peptides (73) circulating in CeD sera but further studies are required to determine the significance of these associations with disease pathogenesis. In recent work in mice, viral infection led to a break in oral tolerance to dietary proteins (40). Some reoviruses can promote a proinflammatory phenotype in mouse dendritic cells (DCs) which lose their capacity to promote tolerance toward food antigens and cause a pathogenic T cell response instead. Reovirus infection causes increased signaling by type 1 interferons and increased expression of the transcription factor interferon regulatory factor 1 (IRF1) which can block the conversion of T cells into regulatory T cells (Tregs) and promote a proinflammatory TH1 response to dietary antigens, respectively. Supporting relevance in humans, patients with CeD tended to have higher anti-reovirus antibody titers. Importantly, reovirus infections are often silent or asymptomatic in humans and a large proportion of the population is exposed to self-limiting gastrointestinal infections during childhood. The findings provide a mechanistic explanation that links an apparently innocuous virus with the loss of tolerance to a common food antigen. More research is required to unravel the significance of viral, bacterial or other microbial host interactions or infections in the development of CeD.

Antibiotics and Delivery Mode

Early case-control studies reported a link between prior antibiotic use and subsequent CeD development in both adults (74) and children (69). Similarly, children with CeD were more likely to have been born by cesarean section (c-section) (75). A large

TABLE 1 | Environmental factors potentially associated with CeD development.

Risk factor	Effect on CeD risk	Studies (refs)
GLUTEN INGESTION		
Age at gluten introduction (timing)	No association	Systemic review with meta-analysis (32); review (33); RCT (34).
Amount of gluten introduction	Conflicting data	A case-control study showed the amount of gluten consumed until 2 years of age increased CeD risk (35); An RCT in HLA-at risk infants with low dose of gluten (100 mg) introduced at 4–6 months showed overall no effect on risk (36).
INFECTIONS		
Infections (overall)	Increased	Increased risk of CeD especially with many infections (10 or more) up to 18 months of age (37).
Infections (gastrointestinal)	Increased	Gastrointestinal infection increased CeD risk autoimmunity by 33%. Risk was reduced in children vaccinated against rotavirus (38).
Rotavirus	Increased	In Sweden rotavirus vaccination has not reduced CeD prevalence (39).
Reovirus	Past infection associated with CeD and possible mechanism established	Higher prevalence of reovirus antibodies in CeD patients vs. controls; Reovirus infection may impair development of oral tolerance (40).
Helicobacter pylori	Conflicting data	Inverse relationship with CeD (41–43); positive or no association with CeD (44, 45).
PERI-NATAL FACTORS		
Season of birth	Increased risk if born in summer	Multiple populations assessed in different studies (38, 46–48).
Elective cesarean section	No association	Multiple populations assessed in different studies e.g., Norwegian Mother and Child (MoBa) Cohort Study (49), TEDDY cohort (50) and others (51).
Geographic location	Possibly increased with northern latitude (single study)	National Health and Nutrition Examination Survey (NHANES) database; CeD more common in northern compared to southern latitudes (52). However, exceptions to the north-south gradient exist e.g., high prevelance of CeD in Northern Africa (26), (53) and Sardinia (16).
Socio-economic status	Increased risk with higher SES	Unclear if due to biological effect e.g., hygiene hypothesis (31) or if due to differences in health seeking behavior (54).
Maternal gluten consumption	No association	TEDDY cohort; mother's intake of gluten in late pregnancy was not associated with risk of celiac disease in offspring (55).
MEDICATIONS		
Proton Pump Inhibitors (PPI)	Increased	Prior use of PPI strongly associated with CeD: OR 4.79; 95% CI 4.17-5.51) (56).
Antibiotics	No increased risk	Use of the most prescribed antibiotics during the first 4 years of life was not associated with the development of autoimmunity for T1D or CeD (57).
Maternal iron supplementation	Conflicting data	Increased risk in MoBa cohort (58) but not replicated in TEDDY cohort (59).
Vitamin D	No association	Maternal or neonatal vitamin D status not related to the risk of childhood CeD (60).

case-control study found that while emergency c-section was not associated with later CeD development, elective cesarean delivery was (76). However, conflicting data has been reported. For instance, no link between increased CeD risk and antibiotic use during the first 6 months of life (68) or antibiotic use during pregnancy (77) was found. The Environmental Determinants of Diabetes in the Young (TEDDY) study is a multicenter observational cohort study that aims to identify environmental factors associated with T1D and CeD in children at HLA risk followed from birth (78). It found no association between antibiotic use and CeD autoimmunity during the first 4 years of life (57) or between delivery by c-section and increased CeD risk (50). Similarly, large observational studies found no link between c-section and CeD development (49, 69, 79). Finally, a large register-based study, that included children from two independent cohorts, found that birth delivery mode was not associated with increased risk of diagnosed CeD (51). Although the data is conflicting, the potential links between early events that can alter the microbiota composition, such as antibiotic use or birth delivery mode, and later CeD implicate a role of the microbiome in disease development.

THE MICROBIOME IN CELIAC DISEASE

Microbial colonization occurs at birth and shapes the development of the mucosal and systemic immune system and the intestinal barrier. These host-microbe interactions continue throughout life, and a disruption of these interactions, through altered bacterial composition or functions, have been hypothesized to increase the risk of a range of autoimmune or inflammatory diseases such as CeD. Altered microbiota composition in patients with CeD may represent an environmental modifier of CeD development.

An early study described the presence of rod-shaped bacteria in duodenal biopsies of Swedish children with CeD born during the epidemic, which weren't observed in biopsies of control children (80), or in children born following the epidemic (81). The bacteria were subsequently identified as *Clostridium* spp, *Prevotella* spp, and *Actinomyces* spp, and their presence was suggested to be a risk factor for CeD that contributed to the increase in disease incidence in Sweden from 1985-1995 (81). Subsequent clinical studies have described differences in both fecal and duodenal microbial composition

in children and adults with active compared with treated CeD, or healthy controls (82). While no specific microbial signature has been described for CeD, many groups have described increases in the proportions of *Bacteroides* and members of Proteobacteria, and decreases in *Lactobacillus* and *Bifidobacterium* (83, 84). In addition, CeD patients suffering from persistent symptoms were shown to have increased abundance of Proteobacteria compared to those who were asymptomatic (85). While these studies suggest an association between altered microbial composition and development of CeD, studies exploring mechanisms and causality are lacking. Moreover, whether alterations in the microbial composition are a cause or consequence of small intestinal inflammation has not been fully elucidated.

Recent studies have suggested that the microbiota from CeD patients may harbor more pathogenic or pro-inflammatory bacteria. Reports of CeD diagnosis following *Campylobacter jejuni* infection (86) suggest that bacterial infections could precede CeD development. *Escherichia coli* clones isolated from CeD patients expressed a higher number of virulent genes compared to those isolated from healthy controls (87). Similarly, the presence of virulent genes were higher in *Staphylococcus* spp and in *Bacteroides fragilis* strains isolated from CeD patients compared to healthy controls (88, 89). Importantly, strains isolated from CeD patients were more pro-inflammatory *in vitro* and stimulated altered DC morphology, characteristic of DC maturation, increased pro-inflammatory cytokine production, and altered epithelial barrier integrity. Similarly, *Neisseria flavescens*, a member of Proteobacteria, was identified in the duodenum of active CeD patients but not from control subjects and induced an inflammatory phenotype in human and murine DCs (90).

In contrast to the above studies, bacterial infections may also protect against CeD development. Some studies indicate an inverse relationship between the presence of *Helicobacter pylori* and CeD in both adults and children (41–43) whereas other studies have shown a positive or no association (44, 45). Mechanisms underlying this association have not been elucidated and inconsistencies across studies may relate to differences in techniques used to determine *H. pylori* status or *H. pylori* virulence. Less virulent strains may exacerbate the mucosal response in CeD whereas more virulent strains may provide protection against CeD (45, 91).

Functional differences in the microbiota could also affect metabolic processes important in CeD pathogenesis. The gastrointestinal tract harbors diverse bacteria that participate in gluten metabolism *in vitro* and this may differ between healthy individuals and those with CeD (92–94). As most studies have measured microbial composition in active or treated CeD compared to healthy controls it is difficult to determine whether functional differences are present prior to disease onset.

To gain insight into the potential role of microbial factors in CeD development, previous studies have profiled the fecal microbial composition of genetically at-risk children. High-risk children were shown to harbor a different microbiota compared to children who were at low genetic risk for CeD (95, 96), suggesting that the high-risk genotype may influence early gut microbiota composition. Infants at the highest risk for CeD had a higher prevalence of enterotoxigenic *E. coli* compared to those at low or intermediate risk for CeD (97). In addition, in a cohort of 164 infants, those at risk for CeD had lower numbers of *Bifidobacterium* spp and *B. longum* and increased numbers of *B. fragilis* and *Staphylococcus* spp. The differences in *Bacteroides* and bifidobacteria were attenuated by breastfeeding (98). At-risk children that later developed CeD were recently shown to have an altered microbial trajectory that coincided with immune changes. These changes were suggestive of a "premature maturation" of the gut microbiota in children who went on to develop CeD (99). On the other hand, the fecal microbiota of at-risk infants who went on to develop CeD was similar at 9–12 months to those infants that remained healthy by the age of four (100). Whether the duodenal microbial composition or function is altered in at-risk individuals that go on to develop CeD needs to be investigated further in larger clinical trials.

Diet and environment also determine gut microbiota composition (101, 102), highlighting the complexity of delineating the influence of genotype and environment on shaping the microbiota. Larger clinical trials where both the composition and function of the microbiota is studied in at-risk individuals and followed over time are needed to help understand gene-microbe interactions in CeD development.

THE ROLE OF GENETICS IN DISEASE DEVELOPMENT

While environmental factors are important for CeD development a notable feature of CeD is its high heritability and strong HLA association (103). This strong genetic association reflects the central role of CD4+ T cells as the HLA molecules associated with CeD bind specific gluten peptides that activate T cells (104). Ninety percent of Caucasian CeD patients possess the HLA-DQ2.5 haplotype (encoded by the DQA1*05:01 and DQB1*02:01 alleles) either in *cis* or *trans* positions, and the remaining carry either HLA-DQ8 (encoded by the DQA1*03:01 and DQB1*03:02 alleles), HLA-DQ2.2 alone (encoded by the DQB1*02:02 allele) or HLA-DQ7 alone (encoded by the DQA1*05:01 allele). Less than 1% of CeD patients lack these HLA haplotypes (105) and their absence can be exploited in the clinical setting to assist in excluding a diagnosis of CeD.

A "gene-dose effect" related to the number of copies of the DQB1*02 allele has been reported to affect CeD risk, clinical phenotype and patient responses to a T cell targeted therapy. The presumed basis for this effect is that gluten presented by APCs in HLA-DQ2.5 homozygous (i.e., two copies of DQB1*02) individuals can induce at least a 4-fold higher T-cell response compared with gluten presented by APCs in HLA-DQ2.5 heterozygous (i.e., one copy of DQB1*02) individuals (106). The CeD risk in HLA-DQ2.5 homozygous patients is ∼2.5 and 5 times that conferred by HLA-DQ2.5 heterozygosity and

lower risk HLA groups, respectively (107). A prospective Italian study (Celiac Disease and Age at Gluten Introduction study; CELIPREV) followed newborns with a family history of CeD and showed the risk of CeD autoimmunity (positive CeD-serology panel) at 10 years of age was far higher among children who were HLA-DQ2.5 homozygous (or who had two copies of DQB1*02 than among those who were HLA-DQ2.5 heterozygous or HLA-DQ8 (38 vs. 19%, $P = 0.001$), as was the risk of overt CeD (26 vs. 16%, $P = 0.05$) (34). In this cohort, 80% of those in whom CeD developed did so during the first 3 years of life. In the TEDDY study following 6403 US and European genetically at risk children at for CeD, the risks of CeD autoimmunity and confirmed CeD by age 5 were 11 and 3%, respectively in the heterozygous children and 26 and 11%, respectively, in those who were homozygous (108).

In addition, HLA-DQ2.5 homozygosity has been associated with a more severe CeD phenotype with earlier disease onset, greater villous atrophy, diarrhea, and lower hemoglobin at presentation, and a slower rate of villous healing on a GFD (109), plus a higher rate of refractory (non-responsive) CeD (110). In a recent clinical trial of an immunotherapy targeting gluten-specific CD4+ T cells, CeD subjects who were HLA-DQ2.5 homozygous were more likely to experience gastrointestinal symptoms following systemic administration compared to those who were heterozygous (111).

The contribution of non-HLA genes to CeD risk susceptibility is much less strong (OR <1.5) than the HLA-associated haplotypes (OR >5) but collectively are significant. More than 70 candidate genes in over 40 non-HLA loci have been implicated in CeD heritability (112–117). These loci encode proteins involved in a range of immune pathways affecting T and B cell activation, chemokine receptor activity and cell migration, cytokine binding, thymic differentiation of CD4+ and CD8+ T cells, stress pathways and innate immunity. Only one gene has been shown to be gut specific (*RGS1*), underscoring the systemic nature of immune dysregulation in CeD (114). To date there is no evidence to implicate specific alleles encoding gastrointestinal proteases or tTG. Mirroring the frequent disease co-occurrence, there is substantial overlap between genetic risk factors for CeD and those of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and T1D (112, 115, 118, 119). Furthermore, despite a much weaker clinical association with CeD, there is overlap of genetic risk loci for inflammatory bowel disease such as Crohn's disease (18, 120). Intriguingly, 90% of the identified risk loci map to non-coding regions such as promoter regions, enhancers or non-coding RNA genes, suggesting that regulation of gene expression rather than changes at the protein-coding level are more important for CeD susceptibility and development (121).

A KEY ROLE FOR T CELLS IN PATHOGENESIS

The role of CD4+ helper T cells in CeD was confirmed with the isolation of pro-inflammatory gluten-specific CD4+ T cells from intestinal tissue of CeD patients (122) (**Figure 1**). These pathogenic T cells have a Th1 phenotype characterized by production of IFN-γ and TNF-α (123) and almost all are HLA-DQ2- and/or DQ8-restricted (122, 124, 125). Gluten peptides that have been post-translationally modified by the enzyme tTG in a process called deamidation can effectively activate these T cells (126, 127). Deamidation converts specific glutamine residues to glutamate and this modification enhances the gluten peptide's binding affinity to disease-associated HLA dimers (128–130). Deamidation is crucial in converting poorly immunogenic wild-type gluten peptides to highly immunogenic antigens for CD4+ T cells. The structural requirements that generate effective binding of gluten peptides to HLA-DQ2 or DQ8 and T cells via the T cell receptor (TCR) have been further elucidated in structural studies (131, 132) and assessment of the biased use of TCR genes (133, 134).

Studies of T cells isolated from the intestine of CeD patients, or from their blood after short-term oral gluten challenge, have been used to define the gluten peptides (specifically, the T cell epitopes) immunogenic in CeD. Most studies have focused on the 90% of CeD patients who are HLA-DQ2.5 and a range of immunodominant T cell epitopes have been defined (135). Less is known about the nature of the T cell response to gluten in CeD patients without HLA-DQ2.5. The most immunogenic gluten epitopes for CeD patients with HLA-DQ2.5 after wheat ingestion reside in α-gliadin and ω-gliadin (136). Much of the field has focused on the immunogenicity of T cell epitopes in α-gliadin, specifically those encompassed within a protease resistant 33mer (137). However, gluten challenge studies show that the most immunogenic peptides induced by gluten ingestion depends on whether wheat, rye or barley is ingested, and that a sequence from ω-gliadin (encompassing the T cell epitopes DQ2.5-glia-ω1 and DQ2.5-glia-ω2) is dominant irrespective of which grain is consumed (136). Despite the multitude of immunogenic peptides, just three peptides from wheat and barley appear to recapitulate most of the response to gluten in CeD patients with HLA-DQ2.5. Interestingly, after oat ingestion, about 8% of CeD patients have detectable T cells specific for avenin peptides that share close sequence homology with barley hordein, suggesting that cross-reactive T cells may mediate immune responses following oats ingestion in some CeD patients (21).

While early work suggested T cells from children with CeD displayed a different pattern of reactivity to gluten compared to adults (138), more recent studies show gluten-specific T cells in blood induced by oral wheat challenge (139), or expanded from the small intestine during active disease (140), share the same specificity for deamidated, immunodominant T cell epitopes across all ages. The same gluten-specific T cell clonotypes persist in patients' blood and intestinal tissue up to several decades and share the same TCR gene use motifs in CeD patients from Norway, Finland and Australia (134, 139, 141, 142). Their stability over such long periods of time may be maintained by ongoing gluten exposure as inadvertent gluten intake is common in CeD even when a strict gluten-free diet is attempted (143, 144).

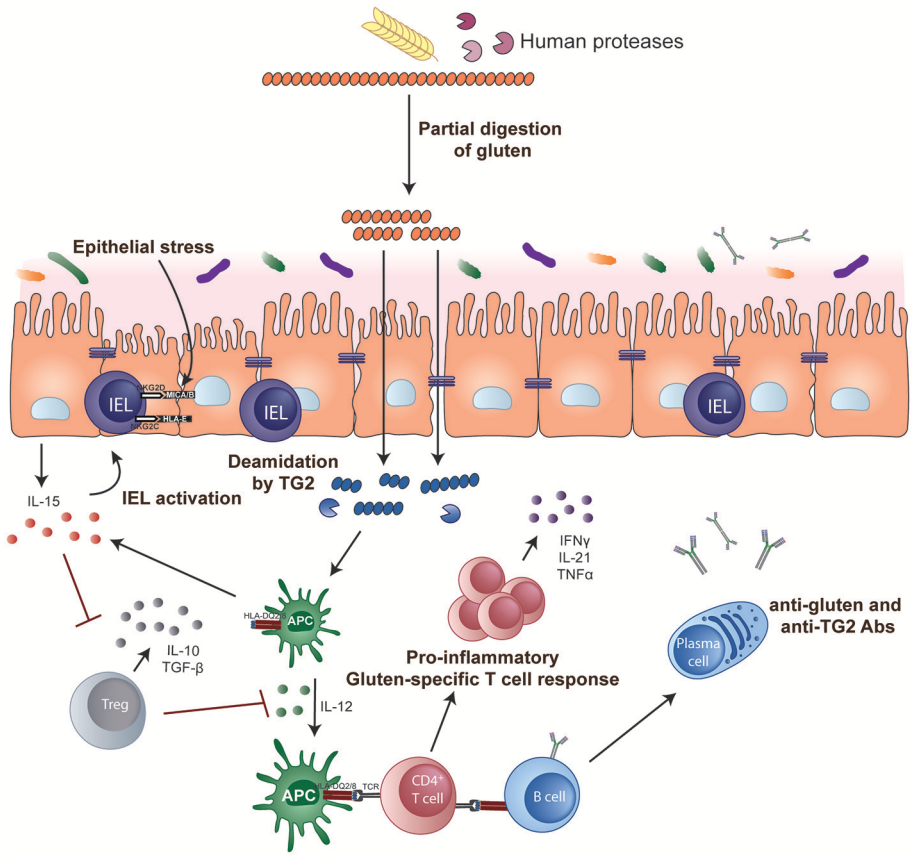

FIGURE 1 | Key steps in CeD pathogenesis. Gluten peptides containing T-cell epitopes resist gastrointestinal degradation. tTG catalyses the deamidation of gluten peptides, which can then bind more efficiently to the disease-relevant HLA-DQ molecules on APCs. Activated gluten-specific CD4+ T cells secrete a variety of pro-inflammatory cytokines such as IFN-γ and IL-21 that contribute to the intestinal lesion and promote activation of IELs and stimulate B-cell responses. Activated IELs transform into cytolytic NK-like cells that mediate destruction of enterocytes expressing stress signals. IL-15 renders effector T cells resistant to the suppressive effects of Tregs and, in the lamina propria, endows mucosal DCs with inflammatory properties promoting pro-inflammatory responses and preventing Treg differentiation.

ANTIBODIES AND B CELLS IN CELIAC DISEASE

Measurement of tTGA is a useful screening test for CeD as the titer reflects disease activity caused by gluten, however a direct role in disease pathogenesis is less clear. As tTGA are anti-angiogenic they may contribute to some extra-intestinal manifestations of CeD (145). As tTGA are detectable in intestinal tissue prior to the typical enteropathy of CeD and predict future disease onset, antibody production is likely to occur early in disease development (146). However, approximately 50% of children with positive CeD serology normalize their levels despite ongoing gluten exposure, suggesting that gluten immunity that leads to a pathogenic response is not necessarily fixed once it has commenced (147). Supporting this concept is the observation that almost 20% of adults with CeD diagnosed during childhood who elect to resume gluten intake have no evidence of active disease (148).

Production of tTGA appears reliant on T cells, as antibody formation to tTG and DGP is strictly dependent on the presence of CeD-associated HLA types as well as gluten. This supports the idea that tTG-specific B cells internalize tTG in complex with gluten peptides and present gluten-derived peptides to gluten-specific T cells, effectively amplifying the T cell response. These T cells then provide the required "help" to the B cell, resulting in production of tTGA and DGP antibodies (149, 150).

In active CeD, a large number of plasma cells can be found in the intestinal lesion and tTG-specific plasma cells made up a large proportion of them (5–25%) (151). There is much that still needs to be understood about how these antibody producing cells are selected and mature. tTG can form covalently linked multimers with itself that readily bind gluten peptides and can be taken up by tTG-specific BCR transduced cells and activate gluten-specific T cells with increased capacity compared to tTG monomers (152). Immunoglobulin expressed on B cells could act as substrates for tTG, in particular IgD, resulting in BCR/tTG cross-linking (153). As B cell epitopes are in close proximity to immunodominant T cell epitopes (154) and react to a higher degree to deamidated peptides (155) future work needs to examine the B cell

and T cell interaction during the gluten-specific immune response.

THE DEVELOPMENT OF INTESTINAL VILLOUS ATROPHY

The events that culminate in the histological changes of CeD are incompletely understood. Gluten T cell epitopes cluster in regions of high proline making them resistant to the effect of gastrointestinal proteases (156). These peptides may pass across the epithelium via transcellular (157–159) and paracellular (receptor or antibody mediated) pathways (160). Increased epithelial permeability, a feature of active CeD, may be mediated by a direct effect of gliadin acting via the chemokine receptor CXCR3 in intestinal epithelial cells on tight junctions (160). DCs are presumed to play a major role in presentation of gluten peptides to CD4+ T cells (161), however little is known about their identity, where this presentation occurs, and the extent to which this role is undertaken by gluten-specific B cells. Activated CD4+ T cells produce large amounts of IFN-γ, that may induce cytotoxicity of intraepithelial lymphocytes (IELs), as well as IL-21, which plays a role in T-cell-dependent B cell responses (162). IL-17 producing CD4+ T cells have been reported in untreated CeD (163) but their role and that of IL-17A production in CeD pathogenesis is less clear (162, 164). IL-15 and IFN-α feature prominently in the inflamed tissue in CeD patients (165, 166). IELs are believed to play an important effector role in mediating destruction of enterocytes in CeD in a TCR-independent manner. When activated by stress signals on intestinal epithelial cells such as HLA-E and MIC-A (167), IELs express high levels of NK activating receptors such as NKG2D and CD94/NKG2C and adopt a cytolytic phenotype capable of destroying enterocytes (168). IL-15 plays a key role by upregulating the activating NKG2D receptor and acting as a co-stimulatory molecule. The effect is to 'license' cytotoxic IELs with the ability to kill intestinal epithelial cells expressing the stress-induced MIC molecules. Adaptive immunity to gluten and epithelial stress where cytotoxic IELs have acquired an activated NK cell phenotype may both be required for villous atrophy to develop in CeD (169).

The causes of epithelial stress that trigger IEL activation and transformation in CeD are not known. Some hypothesize it is driven by gluten itself or other stimuli such as those resulting from a microbe-host interaction (**Figure 2**). The innate immune system is a pre-programmed form of host defense that responds rapidly to stimuli. Responses are triggered when pattern recognition receptors, for example, toll-like receptors on macrophages, bind molecules with conserved structures. An innate immune stimulatory effect of a gliadin sequence (A-gliadin p31-43) has been reported, but this work has not been replicated (170). No other gluten peptides activating innate immunity in humans have been defined. Gliadin may function as a stress signal for the activation of *MICA* expression only in the initial stages of disease and decline to baseline levels once the inflammatory lesion is established. In contrast to the critical role of HLA-restricted, gluten-specific CD4+ T cells in CeD pathogenesis, the relative contribution of innate immunity to disease has not been established in genetic or functional studies and further research in this area is required.

HOW DOES LOSS OF TOLERANCE TO GLUTEN DEVELOP?

Although gluten consumption is common in the Western diet most individuals who possess HLA susceptibility for CeD develop immunologic tolerance to it as they do for other food proteins. Why a small percentage fail to develop or lose tolerance to gluten remains unresolved. Tregs are an important immune

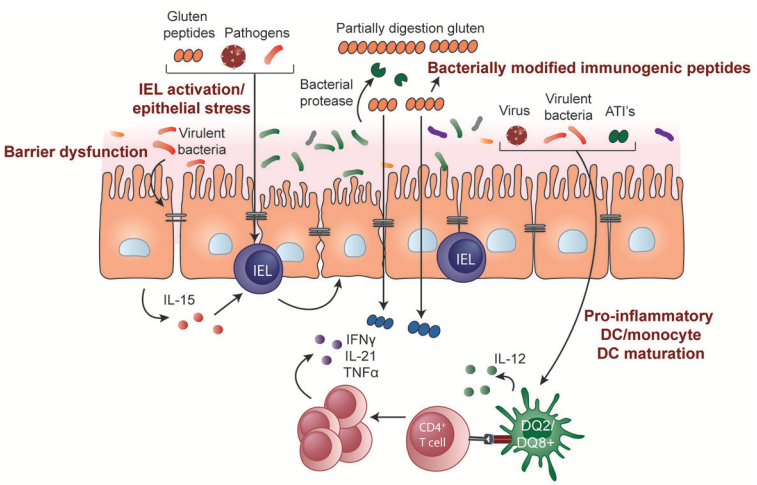

FIGURE 2 | Potential role of microbes and environmental triggers in CeD pathogenesis. Microbes that include both commensals and opportunistic pathogens may contribute to the development of CeD by influencing gluten peptide digestion, intestinal barrier function, epithelial cell stress, or IEL activation/upregulation through IL-15 regulation. Pathogenic bacteria, viruses, and non-gluten components of wheat, such as amylase-trypsin inhibitors (ATIs), may also induce DC maturation and proinflammatory cytokine production, modulating the induction of CD4+ T-cell responses.

component contributing to intestinal homeostasis and when functioning normally inhibit pathogenic responses to dietary antigens and help maintain oral tolerance. This role suggests they may be important in CeD, however identifying and studying this cell population has been limited by the technical challenge of identifying a population of cells truly gluten-specific and functionally suppressive *in vivo*. These challenges have meant that studies of Treg function in CeD have been conflicting, with some indicating reduced suppressive function (171–173) and some showing it is retained (174, 175). Further studies that definitively isolate true gluten-specific Tregs and assess their function in CeD are needed.

Interestingly, the immunosuppressive cytokines IL-10 and TGF-β can be detected in high amounts in the CeD intestinal lesion but appear insufficient to counter active disease (176, 177). The high levels of pro-inflammatory cytokines such as IFN-γ and IL-15 may render this anti-inflammatory response inadequate (178, 179). IL-15 is a pleiotropic cytokine that may promote inflammation through several pathways, including driving the accumulation of cytotoxic IELs in the CeD lesion, interfering with the suppressive activity of Tregs (175), impairing TGF-β signaling (176), or allowing activation of disease-specific CD4+ T cells (180). IL-15 also drives the expansion of aberrant IEL clones that can lead to the development of enteropathy-associated T cell lymphoma. The multiple pro-inflammatory effects of IL-15 make it a rational target for therapeutic blockade, and clinical trials of anti-IL-15 biologic agents are underway in refractory CeD (181).

Additional insights into molecular pathways altered in loss of tolerance may be gained by study of gene expression profiles in CeD patients. Analysis of blood-derived non-gluten stimulated CD4+ T cells in CeD showed significant upregulation of the IFN-γ gene and reduced expression of a network of BACH2 regulated genes (182). BACH2 is a transcription factor that plays an important immunoregulatory role in inhibiting T effector cell development and promoting development of Tregs. Advances in techniques such as RNAseq now affords the opportunity to analyze multiple transcriptional and phenotypic features of antigen-specific effector T cells and Tregs to provide insights at the single cell level into these heterogeneous populations.

The findings on Treg numbers in the CeD lesion suggest that the defect in tolerance is not simply due to defects in numbers or recruitment of suppressive cells, but that the pro-inflammatory environment characteristic of CeD overcomes the tolerogenic milieu that normally maintains balance and inhibits abnormal immune responses. However, further studies on disease-relevant Tregs from the small intestine of CeD patients that assess antigen-specificity, function *in vivo* and the factors that impact their function are required.

FROM PATHOGENESIS TO CLINICAL CARE

Insights From Animal Models

Modeling CeD has been a challenge as no single animal model that encompasses all elements of disease exists. As a result, mouse models have played a limited role in the development or pre-clinical testing of new therapies (183) and have more often been used to investigate specific mechanisms related to disease pathogenesis (184). The transfer of gluten-specific T cells into immunodeficient mice have been used to study the role of CD4+ T cells in mediating tissue damage (185, 186). Transgenic mouse models have also been used to investigate specific cytokines or genetic components in CeD pathogenesis. For example, mice over expressing IL-15 in the lamina propria (165) or in the epithelium (180) have shed light on the role of innate mediators in the development of the intestinal lesion in CeD. Mice that express human HLA-DQ2 or -DQ8 develop gluten-specific T cells and some innate immune activation following sensitization to gliadin with an adjuvant. However, they do not progress to full blown gluten-induced enteropathy (183, 187–189), emphasizing the importance of additional genetic, immune, or environmental factors in triggering tissue destruction in CeD. This lack of spontaneous loss of tolerance to gluten in transgenic mouse models can be taken advantage of and utilized to better understand environmental factors that participate in the loss of tolerance to gluten. For example, the mechanisms through which microbes contribute to CeD development can be studied by manipulating the microbiota composition or exposing transgenic mouse models to certain bacteria.

In mice expressing human HLA-DQ8 the composition of the gut microbiota was found to influence the degree of gluten-induced immunopathology (190). Mice harboring a limited microbiota devoid of Proteobacteria and opportunistic pathogens were protected from gluten-induced pathology and immune responses. However, this protective effect was lost when these mice were supplemented with an enteroadherent strain of *E. coli* that was isolated from a CeD patient. Similarly, treatment of specific pathogen free mice with vancomycin increased Proteobacteria levels, including *Escherichia*, and led to more severe gluten-induced pathology. While the mechanisms remain elusive, the results provide a proof-of-concept that microbes could alter how a host responds to gluten and could therefore be targeted as a prophylactic approach.

Gnotobiotic mice, or mice colonized with known microbes, provide a model where the impact of specific bacteria on gluten-mediated responses *in vivo* can be studied in a controlled environment. Studies of mice colonized with bacteria isolated from the duodenum of CeD patients or from healthy controls have shown that bacteria participate in gluten metabolism *in vivo* (94). Interestingly, the immunogenicity of the end products generated by bacterially-mediated gluten digestion differed depending on the type of bacteria. Following human protease digestion, elastase from *Pseudomonas aeruginosa* generated highly immunogenic gluten peptides that could strongly activate gluten-specific T cells from human CeD patients. These peptides were better able to translocate the epithelial barrier, potentially facilitating immune cell-peptide interactions. Conversely, gluten peptides produced following digestion by human proteases or by elastase from *P. aeruginosa* were detoxified or degraded by *Lactobacillus spp*, a core member of a healthy microbiome. The continued use of gnotobiotic models will be critical for understanding how microbes or pathogens may interact with the

host and/or gluten to contribute to CeD pathogenesis (**Figure 2**). Importantly, these models can also be used to test microbiota-targeted therapies for CeD.

Can Celiac Disease Be Prevented?

Population studies have provided important insights into environmental factors associated with CeD but are unable to establish true causality or mechanism. If factors that impact CeD risk can be identified and modified, then prevention of CeD may be possible. Randomized controlled trials (RCTs) do allow a controlled assessment of how a factor impacts CeD risk and several have now been undertaken or are underway in genetically at-risk infants or children (**Table 2**). The results have sometimes differed from assumptions made in population studies, underscoring the importance of running well designed and controlled intervention studies and undertaking research to examine mechanism.

Two independent RCTs assessed whether low amounts of gluten can prevent genetically at-risk children from developing CeD. The idea of a "window of opportunity" during which gluten could be introduced in small amounts to induce tolerance was based on previous experience from the Swedish epidemic and supported by a prospective study showing infants exposed to gluten either early (<4 months) or late (>7 months) were at an increased CeD risk (63). The optimal window was proposed between 4 and 6 months, preferably during ongoing breast-feeding (191). In the multicenter PreventCD study, almost 1,000 genetically at-risk children with at least one first-degree relative with CeD were randomized to a double-blind, placebo-controlled dietary-intervention to receive 100 mg of gluten daily or placebo from 16 to 24 weeks of age (36). Neither breast-feeding nor the introduction of small quantities of gluten at 16–24 weeks of age reduced the risk of CeD by 3 years of age in this group of high-risk children (36). Published in the same journal, the CELIPREV group randomised over 800 newborns who had a first-degree relative with CeD to have dietary gluten introduced at either 6 or 12 months (34). The results from the CELIPREV study concorded with the results from the PreventCD study, showing that neither the delayed introduction of gluten nor breast-feeding modified the risk of CeD among at-risk infants (34). Although CeD was not prevented by delaying the introduction of gluten, it was associated with a delayed onset of disease (34). Since these two RCTs were published, other clinical trials have been performed or are currently being conducted.

Another potential approach to CeD prevention is through modification of the host-microbe interactions in at-risk individuals. Trials to assess the impact of probiotic supplementation in preventing CeD in genetically at-risk children are underway (**Table 2**). If successful, they would support the idea that altered function of the microbiome is a major event underpinning the development of CeD, and that preventing dysregulated host-microbe interactions may be of prophylactic benefit. Furthermore, a better understanding of the mechanisms through which microbes contribute to CeD development can provide further rationale and a more targeted approach for microbiota-modulating preventative strategies.

Improving the Diagnosis of Celiac Disease

While villous atrophy remains the cornerstone of CeD diagnosis there is the growing realization that this "gold-standard" has limitations. For example, results are affected by the number of samples collected and how the biopsies are oriented and reported (192–195). Ultra-short CeD where villous atrophy is present only in the duodenal bulb and "mild enteropathy CeD" where villous atrophy is absent in the setting of positive CeD serology both present diagnostic challenges and highlight potential shortcomings of histology (196, 197). Improvement in the quality of serological testing for CeD and the requirement for specific HLA genotypes for CeD to develop has meant that a serogenetic approach to CeD diagnosis is appealing and may be sufficient for diagnosis in the right clinical situations (2, 198). As expeditious treatment of CeD may avoid or reduce the risk of many CeD-associated complications such as impaired bone density and stunted growth in children, improving early diagnosis remains a clinical and research priority.

In recent years the high rate of community adoption of the GFD, including in children (199), has compounded the challenge of CeD diagnosis as the accuracy of current serological and histological approaches depend on active gluten intake. In order to make a CeD diagnosis reintroduction of dietary gluten, generally for several weeks to months, is recommended prior to testing but patients are often reluctant to undertake this and for those that do many fail to tolerate it. As the serologic and histologic response to gluten challenge is highly heterogeneous the optimal duration of gluten challenge required for definitive diagnosis of CeD remains uncertain (200–202). Immune diagnostics that measure the gluten-specific immune response target a fundamental component of CeD and may overcome the limitations of current diagnostics. The use of tetramers (203) or cytokine release assays (204) to identify gluten-specific T cells induced in blood after short-term oral gluten challenge is highly sensitive and specific for CeD (205). Diagnostics that are accurate with limited or even no gluten exposure such as tetramer-based detection of gluten-specfic T cells (206) are particularly appealing to clinicians and patients as they may avoid the need for prolonged gluten challenge prior to testing with serology and histology. Large multi-center validation studies to confirm the accuracy of assessing disease-specific T cells as a CeD diagnostic are required, and if successful, may force a re-think of how CeD should be classified. Arguably, CeD may be better defined by the HLA-linked, T cell mediated systemic response to gluten rather than histologic changes in the proximal small intestine or circulating antibodies that indirectly reflect disease activity.

Improving the Treatment of Celiac Disease

While adherence to a strict and lifelong GFD still remains the single proven and available treatment for CeD, it is for many patients complicated, onerous, and expensive. In adults with CeD, daily consumption of as little as 50 mg of gluten, equivalent to that contained in 1/100th of a slice of standard wheat bread,

TABLE 2 | Prospective trials in infants/children looking at factors impacting celiac disease development.

Study	Design	Finding	Reference or clinical trials identifier
Prevent Coeliac Disease Study (PreventCD)	International double-blind placebo controlled RCT: 100 mg of gluten daily or placebo from 16 to 24 weeks of age	Neither breast-feeding nor introduction of small quantities of gluten at 16–24 weeks of age reduced the risk of celiac disease by 3 years of age in this group of high-risk children	(36)
Celiac Prevention Study (CELIPREV)	Multicenter RCT: Compare introduction of gluten at 6 vs. 12 months	Neither the delayed introduction of gluten nor breast-feeding modified the risk of celiac disease among at-risk infants	(26)
Celiac Disease Prevention With Probiotics Study (CiPP)	Double-blind RCT: Probiotic (*Lactobacillus*) vs. placebo in infants/children aged 2 to 12 years	Completed	NCT03176095
PreCiSe study	RCT: Probiotic vs. placebo vs. GFD from before age of 4 months for 3 years	In progress	NCT03562221

over three months can damage the small intestine (207). A safe gluten "dose" threshold relevant to children with CeD has not been assessed in a controlled trial. Several longitudinal studies in adults with CeD indicate that failure to achieve mucosal healing is common even in those appearing to maintain good dietary adherence over many years (208–213). While healing is considered to be more complete and faster in children with CeD treated with a GFD one study showed 19% had persistent disease activity after 12 months on a GFD (214). Assuming enough time has elapsed on the GFD, persistent mucosal activity may be driven by ongoing, potentially intermittent, gluten exposure (143), such as that inadvertently consumed in contaminated meals when eating out (144). The challenge in maintaining adequately strict gluten exclusion and persistent disease activity is a major driver for research into new therapeutic approaches. While several therapies are under development it is notable that none of them have yet been evaluated in children.

Clinical trials of novel therapies for CeD have increased substantially in recent years but compared to other illnesses such as inflammatory bowel disease the field is still in its infancy. No therapeutic approach for CeD has yet completed Phase 3 clinical trials. An understanding of the optimal goals of treatment and the methods to assess efficacy are an evolving area and have been shaped by the requirements of regulatory bodies such as the FDA. Symptom improvement is now regarded as a key outcome measure and this has driven interest in validating patient reported outcome measures (215) and understanding the basis for gluten-induced symptoms in CeD. A standardized approach to reporting small intestinal histology based on quantitative assessment of morphology (villus height, crypt depth and their ratio) and inflammation (density of intraepithelial lymphocytes) is now commonly employed in CeD clinical trials (192). Confirming adequate dietary gluten exclusion during studies is a major challenge as symptom records, serology, histology, and dietary history are indirect measures of GFD adherence (216). New technology based on the detection of urinary or fecal gluten immunogenic peptides (GIPs) derived from the 33mer peptide in wheat α-gliadin provides objective evidence of dietary gluten exposure (217, 218). In addition to a role in the clinic, it may be a promising tool for evaluating and selecting patients for CeD clinical trials where controlling for inadvertent gluten exposure is important, such as therapies designed to prevent symptoms due to inadvertent gluten exposure (215).

Insight into the molecular mechanisms underpinning CeD pathogenesis provide several opportunities for novel therapeutics development and a range of pharmaceuticals are currently being assessed in pre-clinical and clinical trials (**Table 3**). These can be broadly classified into luminal approaches that aim to quantitatively reduce the load of gluten available to trigger the immune response and qualitative approaches that aim to induce gluten tolerance. A third category, not discussed in this review, encompass immunomodulators (e.g., budesonide, azathioprine), biologics (e.g., anti-IL-15, anti-CD52), and chemotherapy (e.g., cladribine) used to treat refractory CeD (219).

Quantitative approaches include the use of (i) endopeptidase enzymes (glutenases) derived from plants, bacteria or fungi that have a gluten degrading effect, such as latiglutenase (ALV003) (220, 221) and AN-PEP (222) (ii) agents to reduce paracellular passage of gluten i.e., larazotide acetate, an intestinal tight junction regulator that may enhance barrier function (223–225), and (iii) compounds that bind gluten in the gut lumen to reduce absorption, such as the polymer BL-7010 (226). Supplements to the GFD which render small amounts of dietary gluten harmless could substantially improve the quality of life of patients by allowing them to dine out with less fear of adverse effects resulting from contamination by small amounts of gluten. Enzymatic approaches could also be applied during the baking process to reduce gluten immunogenicity (227). Genetic modification of wheat with a variety of targeted techniques such as RNA interference (228) and CRISPR (229) can reduce gluten T cell epitope content and immunogenicity however clinical feeding trials are awaited. The recent publication of the first fully annotated reference wheat genome is an important advance that may help guide targeted approaches (230). Use of protease inhibitors, such as elafin, which is decreased in the mucosa of patients with active CeD has been proposed as it has barrier enhancing and anti-inflammatory effects in gluten-sensitive mice (231).

TABLE 3 | Experimental therapies for celiac disease in pre-clinical or clinical development.

Approach	Proposed mechanism	Phase of development
LUMINAL		
Endopeptidases e.g., latiglutenase, An-PEP	Enzymatic degradation of gluten	Phase 2
Tight junction modulators e.g., larazotide acetate (AT-1001)	Reduce paracellular passage of gluten across mucosa	Phase 2
Transglutaminase inhibitors e.g., ZED 1227	Inhibit conversion of gluten to more immunogenic form	Phase 2
Gluten binding agents e.g., BL-7010	Sequester gluten in the intestinal lumen	Phase 1
HLA-DQ2 blockers	Prevent activation of gluten-specific T cells	Pre-clinical
Non-toxic gluten	Modified or selectively bred cereals devoid of toxicity	Pre-clinical
Inhibition of inflammatory proteases e.g., elafin	Anti-inflammatory effects and improved barrier function	Pre-clinical
TOLEROGENIC		
Peptide-based therapeutic vaccine (Nexvax2)	Epitope-specific targeting of gluten-specific CD4+ T cells	Phase 2
Hookworm (*Necator americanus*)	Immunoregulatory effect of hookworm combined with low-dose gluten exposure	Phase 2
Nanoparticle therapy (TIMP-GLIA)	Nanoparticle encapsulating gliadin delivered intravenously	Phase 1

A phase 2 RCT of latiglutenase taken orally showed it could attenuate small intestinal mucosal injury in CeD patients induced by 2 g of ingested gluten (221). In symptomatic CeD patients following a GFD, latiglutenase reduced symptoms in the subgroup who were seropositive (232), suggesting that gluten exposure was necessary in order to demonstrate a positive effect of the enzyme. In a Phase 2 trial larazotide acetate was shown to reduce symptoms in CeD patients on a GFD better than a GFD alone, but only at the lowest dose of 0.5 mg (225). More studies are required to establish the efficacy of these approaches and how they can be safely used by patients. A controlled gluten challenge will be an important component of the study design in order to demonstrate efficacy and establish the amount of ingested gluten patients can be protected from.

Qualitative approaches aim to establish durable immune tolerance to gluten. One way this might be achieved is by targeting the long-lived population of gluten-specific T cells and deleting or rendering them functionally unresponsive (anergic) and inducing suppressive Tregs (233). As the target T cell population is stable in established CeD (139, 140, 142), it is anticipated these approaches will apply similarly to children as they do in adults with CeD. Phase 1 studies of Nexvax2, a therapeutic vaccine composed of three gluten peptides encompassing immunodominant HLA-DQ2.5-restricted T cell epitopes, initially caused gastrointestinal symptoms similar to those triggered by gluten, however after later administration of Nexvax2 symptoms were no different from those after placebo (111, 234). The recall immune response to gluten was modified in people with CeD receiving Nexvax2. A phase 2 clinical trial of infection with the hookworm *Necator Americanus* combined with a micro-gluten challenge in 12 CeD patients showed immune modifying effects and clinical protection against gluten (235) and a larger controlled study is underway.

CONCLUSION AND FUTURE PERSPECTIVES

Our view of CeD has evolved from a gastrointestinal illness to an immune disease characterized by the presence of specific HLA genes, CD4+ T cells responding to specific gluten peptides, circulating antibodies to tTG and systemic clinical manifestations. Aside from the fact it is driven by an exogenous, dietary antigen, the genetic and immunologic basis for CeD overlaps with that of traditional autoimmune diseases. While HLA susceptibility and wheat consumption and are major determinants of disease development it is apparent that non-HLA genes and a range of environmental factors are important for disease development. Prospective studies have established that the timing of gluten introduction and breastfeeding do not impact the development of CeD. More results from multicenter, prospective longitudinal studies are needed to understand the long-term effects of a high amount of gluten intake and to identify if other environmental exposures might trigger the disease.

Furthermore, although *in vitro* and *in vivo* studies suggests there are host-microbe or gluten-microbe interactions that promote gluten-specific immune responses, larger clinical trials where both the composition and function of the microbiota is studied in at-risk individuals and followed over time are needed to help understand gene-microbe interactions in CeD development. These kinds of studies may provide insight into the microbial events leading to CeD development that could be targeted as preventative or therapeutic strategies.

Finally, understanding how tolerance to gluten is lost in CeD is a fundamental question that needs more study. Insights into disease relevant pathways may come from analysis of the genome and gene expression in CeD, and epigenetic studies are needed to examine the impact of environment on gene expression and disease development. While novel therapies for CeD have not yet been tested in children, emerging studies on the role of environmental factors and the microbiome and how they might impact gluten immunity and tolerance may one day make disease prevention possible. For now, as primary prevention of CeD is a highly attractive, but as yet unrealized goal, the focus must be on driving expeditious diagnosis and treatment in symptomatic children and adults.

AUTHOR CONTRIBUTIONS

JT-D wrote the first draft of the manuscript. DA and HG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Celiac Disease Prevention

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Celiac disease (CD) is a common autoimmune disorder induced by ingestion of gluten in genetically susceptible individuals. Despite the prerequisite for a genetic predisposition, only a minority of the 40% of the Caucasian population that has this genetic predisposition develops the disease. Thus, environmental and/or lifestyle factors play a causal role in the development of CD. The incidence of CD has increased over the last half-century, resulting in rising interest in identifying risk factors for CD to enable primary prevention. Early infant feeding practices have been suggested as one of the factors influencing the risk of CD in genetically susceptible individuals. However, recent large prospective studies have shown that neither the timing of gluten introduction nor the duration or maintenance of breastfeeding influence the risk of CD. Also, other environmental influences have been investigated as potential risk factors, but have not led to primary prevention strategies. Secondary prevention is possible through early diagnosis and treatment. Since CD is significantly underdiagnosed and a large proportion of CD patients are asymptomatic at the time of diagnosis, secondary prevention will not identify all CD patients, as long as mass screening has not been introduced. As following a gluten-free diet is a major challenge, tertiary prevention strategies are discussed as well.

Keywords: celiac disease, prevention, preventive strategies, environmental factors, tertiary prevention

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INTRODUCTION

The incidence and prevalence of celiac disease (CD) have risen over time; this is, in part, due to the current awareness in combination with the advent of highly sensitive and specific serological tests, but it also reflects a true increase in the prevalence of CD (1, 2). The clinical presentation of CD has changed dramatically in the last decades. Patients with atypical or non-specific symptoms often report a delay in diagnosis of CD that may last for years (3) or even worse, CD remains unrecognized and, therefore, untreated (4–6). Untreated disease is associated with long-term complications, such as chronic anemia, delayed puberty, neuropsychiatric disturbances, associated autoimmune disorders, infertility, small-for-date-births, osteoporosis, and, rarely, malignancy and it can reduce the quality of life (7–9). Treatment with a gluten-free diet (GFD) reduces the burden of morbidity and mortality associated with untreated CD. Thus, prevention would be beneficial (10). Prevention is defined as any activity that reduces the burden of mortality or morbidity from disease, taking place at the primary, secondary, or tertiary level (11) (**Table 1**). The purpose of this review is to present the current knowledge of the preventive strategies for CD (**Table 2**).

TABLE 1 | Definition of levels of prevention.

Primary	Secondary	Tertiary
Avoiding the development of a disease	Early detection and treatment	Reducing the impact of existing disease by improved treatment

TABLE 2 | Some possible prevention strategies for celiac disease, as discussed in this review.

Primary	Secondary	Tertiary
• Infant feeding -Breastfeeding -Breastfeeding at the Time of gluten introduction -Timing of gluten introduction -Amount of gluten at the Time of gluten introduction • (Intestinal) infections • Type of delivery • Antibiotics • Microbiota	• Case finding • Screening of high-risk groups • Mass screening	• Optimal adherence to the gluten-free diet • Gluten immunogenic peptides • Dietary interview • Dietary questionnaires • Serology/duodenal biopsies • Additional treatments -Larazotide acetate -Endopeptidases -Desensitization therapy

PRIMARY PREVENTION

Infant Feeding

Theoretically, CD could be prevented by avoiding gluten introduction into the feeding of infants genetically predisposed to CD. However, this is not a realistic strategy, because the strongest genetic predisposing factors for CD, HLA DQ2 and/or HLA-DQ8, are present in about 40% of the Caucasian population. In addition, most of these individuals do not develop CD, since the prevalence of CD is ∼1%. Another reason why avoiding gluten ingestion by a large part of the population is not desirable is that gluten-containing cereals (among others wheat, barley and rye) are important sources of dietary iron, fiber, calcium, folate, and vitamin B12 (12, 13).

Much knowledge about the possible relationship between infant feeding practices and the development of CD has been obtained from "The Swedish epidemic of CD" during the mid-1980s. Between 1985 and 1987, the incidence of CD in Swedish children younger than 2 years of age increased 4-fold, followed by a rapid decline in its incidence around 1995 (14). The occurrence of the epidemic was related to new dietary recommendations: delaying the introduction of all gluten-containing foods to infants until 6 months of age and changes in breastfeeding practices. In Sweden, the incidence of CD diminished when earlier introduction of gluten (>4 months) was reintroduced (14). Many retrospective studies have investigated this hypothesis that delayed introduction of gluten leads to CD with conflicting results. Results of observational studies suggested the existence of a "window of opportunity" for primary prevention, by introducing gluten between 4 and 6 months of age to reduce the risk of CD (**Table 3**). This and other early feeding practices, such as breastfeeding and breastfeeding at the time of gluten introduction, have been investigated as primary prevention strategies for reducing the risk of CD as well (**Tables 4**, **5**). A systemic review and meta-analysis, which included all of the studies published on this topic between 1966 and 2004, found that breastfed children had a 52% reduction in the risk of being affected by CD compared to those who were not breastfed during the time of gluten introduction (pooled OR 0.48; 95% CI: 0.40 to 0.59) (37). However, all of these studies were observational and retrospective.

Among the prospective studies that have been published, there are two gluten interventional ones, namely PREVENTCD and CELIPREV (15, 16) (**Table 3**):

- PREVENTCD is a multinational, randomized, double-blind, placebo-controlled dietary interventional study involving 944 children who had at least 1 first-degree relative with CD and HLA-DQ2 and/or DQ8. From age 4 to 6 months, 475 participants received 100 mg of vital gluten daily and 469 received placebo. After 24 weeks, intake of gluten was liberalized in both groups. CD serology was measured periodically. Children with elevated levels of CD antibodies and/or with symptoms suggestive of CD were offered small bowel biopsies to confirm the diagnosis. The results showed no significant difference between the groups receiving the early gluten intervention or placebo in the risk of developing CD at the age of 3 years.
- CELIPREV is an Italian multicenter, randomized, interventional study that compared early (at 6 months of age; $n = 297$) and delayed (at 12 months of age; $n = 256$) introduction of gluten into the diet of infants at risk for CD (first-degree relative with CD; HLA-DQ2 and/or DQ8 positivity). The results showed a reduced risk of developing CD by the age of 2 years in those with delayed introduction to gluten at 12 months, but no difference between groups in the risk of developing CD at 5 years of age.

A few of the large, prospective, observational cohort (non-interventional) studies assessing the relationship between infant feeding practices and the risk of CD and/or CD autoimmunity (CDA) pointed out the following (**Tables 3**–**5**):

- The Generation R cohort study, including 1679 genetically susceptible CD children from the general population of Rotterdam, the Netherlands, showed that neither breastfeeding for 6 months or longer nor later exposure to gluten (>6 months) compared to earlier exposure (<6 months) was significantly associated with CDA (21).
- The Norwegian Mother and Child Cohort Study (MoBa) showed that breastfeeding longer than 12 months was associated with a higher risk of CD (22). However, this cohort only considered children with clinically diagnosed CD, so probably missed an important proportion of the children with CD.
- The BABYDIAB, a German cohort study, found no association between the duration of breastfeeding nor gluten introduction before or after 3 months of age and risk of CDA (26).

TABLE 3 | Evidence of the effect of the timing of gluten introduction into the diet of young children and the risk of celiac disease.

First author, year, study	Conclusion
INTERVENTIONAL STUDIES	
Vriezinga et al. (15), PREVENTCD	No significant difference in CD development at 3 years for gluten introduction at 4 vs. 6 months^
Lionetti et al. (16), CELIPREV	No significant difference in CD development at 5 years for gluten introduction at 6 vs. 12 months
Sellitto et al. (17)	No significant difference in CDA* risk for gluten introduction at 6 vs. 12 months
Hummel et al. (18)/Beyerlein et al. (19)**	No significant difference in CD and CDA for different gluten introduction at 6 vs. 12 months
PROSPECTIVE COHORT STUDIES	
Andrén Aronsson et al. (20), TEDDY	No significant difference in CD and CDA for gluten introduction at <17 vs. 17–26 vs. >26 weeks
Jansen et al. (21), Generation R	No significant difference in CDA for gluten introduction at <6 vs. >6 months
Størdal et al. (22), MoBa	Borderline significant difference in CD development at gluten introduction <6 vs. >6 months
Welander et al. (23), ABIS	No significant difference in CD for different times of gluten introduction from 0 to 12 months
Norris et al. (24), DAISY	Significantly more CD with gluten introduction <3 or >7 months vs. gluten introduction between 4 and 6 months.
Ziegler et al. (25), BABYDIAB	No significant difference in CD for gluten introduction ≤3 vs. >6 months
Hummel et al. (26), BABYDIAB	No significant difference in CDA for gluten introduction <3 vs. >3 months
RETROSPECTIVE STUDIES	
Ivarsson et al. (27)	Significantly more CD with gluten introduction >6 months compared to gluten introduction between 4 and 6 months.
Peters et al. (28)	No significant difference in CD gluten introduction at ≤3 vs. >3 months
Falth-Magnusson et al. (29)	No significant difference in CD for different times of gluten introduction
CROSS-SECTIONAL STUDY	
Ivarsson et al. (30), ETICS	Significantly more CD with gluten introduction >6 months compared to gluten introduction between 4 and 6 months.

CD, celiac disease; CDA, celiac disease autoimmunity.
**Celiac disease autoimmunity.*
***Same population.*
^Months of age.

TABLE 4 | Most important studies on the evidence of protection from celiac disease with breastfeeding.

First author, year, study	Conclusion
INTERVENTIONAL STUDIES	
Vriezinga et al. (15), PREVENTCD	No effect
Lionetti et al. (16), CELIPREV	No effect
PROSPECTIVE STUDIES	
Jansen et al. (21), Generation R	No effect
Størdal et al. (22), MoBa	No effect*
Welander et al. (23), ABIS	No effect
Norris et al. (24), DAISY study	No effect
Ziegler et al. (25)/Hummel et al. (26)**, BABYDIAB	No effect
RETROSPECTIVE STUDIES	
Decker et al. (31)	No effect
Roberts et al. (32)	No effect
Ivarsson et al. (27)	Protective
Peters et al. (28)	Protective
Greco et al. (33)	Protective
Ascher et al. (34)	No effect
Falth-Magnusson et al. (29)	Protective
Auricchio et al. (35)	Protective
CROSS-SECTIONAL STUDY	
Ivarsson et al. (30), ETICS	Protective

**Breastfeeding (BF) > 1 year predisposing; **Same population.*

TABLE 5 | Evidence of the effect of breastfeeding at the time of gluten introduction and risk for celiac disease.

First author, year, study	Conclusion
INTERVENTIONAL STUDIES	
Vriezinga et al. (15), PREVENTCD	No effect
Lionetti et al. (16), CELIPREV	No effect
PROSPECTIVE COHORT STUDIES	
Andrén Aronsson et al. (36), TEDDY	No effect
Størdal et al. (22), MoBa	No effect
Hummel et al. (26), BABYDIAB	No effect
Norris et al. (24), DAISY	No effect
RETROSPECTIVE STUDIES	
Ivarsson et al. (27)	Protective
Peters et al. (28)	Protective
Ascher et al. (34)	No effect
Falth-Magnusson et al. (29)	Protective

- The Environmental Determinants of Diabetes in the Young (TEDDY) project is an observational, prospective, cohort study that followed children at genetic risk for type 1 diabetes, wherein development of CD is a secondary outcome. The TEDDY study included 6,403 children with a genetic predisposition to developing CD in the United States, Finland, Germany, and Sweden. The study found that gluten introduction before 17 weeks of age or later than 26 weeks of age was not associated with an increased risk for CDA or CD; however, continuation of breastfeeding more than 1 month after gluten introduction compared with discontinuation of breastfeeding prior to gluten introduction was associated with increased risk of CDA but not of CD (20).

Two systematic reviews and meta-analyses, which included the above prospective interventional studies and large cohort

studies (**Tables 3**–**5**), concluded that the timing of gluten introduction and the duration or maintenance of breastfeeding do not influence the development of CD (38, 39).

Interest in the quantity of gluten at introduction into the diet of infants was also raised based on the results of the Swedish CD epidemic. The evaluation of results of one retrospective observational study indicated that large amounts of gluten (>16 g/day) at the time of first introduction increased the risk of CD (27). The same group of investigators further compared, in the ETIC project, 2 populations born in 1993 and 1997; they found a lower risk of CD in the population born in 1997 who ingested significantly less gluten-containing cereal compared to the population born in 1993 (24 vs. 38 g/day intake, respectively, under the age of 2 years) (30). Also, the Swedish case control study from the TEDDY cohort, in which gluten intake was assessed by dietary questionnaires, found that a high intake (>5.0 g/day) of gluten during the first 2 years of life was associated with an increased risk of CD (36). However, a similar analysis of the data in the international PREVENTCD study showed that the amount of gluten consumed at 11–36 months of age did not influence the risk for CD development (40). Thus, the influence of the amount of gluten intake on CD risk remains a topic of discussion.

In accordance with the results from the above-mentioned studies, ESPGHAN has updated its guidelines for gluten introduction into the diet of young children. The current recommendation no longer suggests introducing gluten between 4 and 6 months of age; rather they recommend that gluten may be introduced into the infant's diet anytime between 4 and 12 completed months of age, since gluten introduction in these infants does not seem to influence the absolute risk of developing CDA or CD during childhood (38).

In addition to gluten and breastfeeding, other environmental factors may be involved in the risk and/or prevention of CD. Identifying and influencing these factors may lead to preventive strategies. Some of these factors are discussed below.

(Intestinal) Infections

Intestinal infections might change gut permeability and lead to the passage of immunogenic gluten peptides through the epithelial barrier, and thus, activate an autoimmune reaction. Many groups have studied the relationship between infections, both viral and bacterial, and the risk of CD, with varying results (**Table 6**). The role of early infections was retrospectively explored in the Swedish population-based incident case referent ETICS study. Having three or more parental-reported infections, regardless of the type of infection, during the first 6 months of life was associated with significantly increased risk of CD, even after adjusting for infant feeding and socioeconomic status (61).

Results of prospective studies are contradictory. Data from the PREVENTCD study showed no significant difference in the cumulative incidence of CD between children with and without parental-reported gastrointestinal infections in the first 18 months of life (15). However, the TEDDY study found that parental-reported early gastrointestinal infections increased the risk of CDA within the following 3 months (HR 1.33; 95% CI 1.11–1.59). This effect was observed particularly in those children with non-HLA-DQ2 genotypes who had been breastfed for <4 months, as well as in children born in winter and introduced to gluten before the age of 6 months (62). In the prospective MoBa study, children with ≥10 infections (respiratory and gastrointestinal) before 18 months of age had a higher risk of being clinically diagnosed with CD compared with children who had ≤4 infections, even after adjustments for antibiotic exposure (63). Viral infections have been suggested to play a role in the development of CD (**Table 9**), and recently, reovirus has been reported as a trigger for the disease, both *in vitro* as well as *in vivo* (60). *In vitro*, reovirus infection induced a disruption of intestinal immune homeostasis and initiated loss of oral tolerance and T-helper inflammatory immunity to dietary antigens. In CD patients anti-Reovirus antibodies were significantly overrepresented in comparison to health controls.

However, this disruption of the immune homeostasis may not be exclusive to reovirus and their role in the development of CD should be studied prospectively.

TABLE 6 | Some of the most relevant studies[#] on infections and the risk of celiac disease or celiac disease autoimmunity.

First author, year, study	Pathogen	Association between CD(A)
PROSPECTIVE STUDIES		
Stene et al. (41)	Rotavirus	Positive
Thevenot et al. (42)	Hepatitis C virus	None
Gravina et al. (43)	Hepatitis C virus	None
Jansen et al. (44)	EBV, CMV and HSV-1	Negative
Karhus et al. (45)	Influenza	None
Dore et al. (46)	Helicobacter Pylori	None
RETROSPECTIVE STUDIES		
Lahdeaho et al. (47)	Adenovirus 12/40	Positive
Vesy et al. (48)	Adenovirus 12, CMV, HSV	None
Kagnoff et al. (49)	Adenovirus 12 Adenovirus 18/echovirus 11	Positive None
Mahon et al. (50)	Adenovirus 12	None
Fine et al. (51)	Hepatitis C virus	Positive
Carlsson et al. (52)	Enterovirus	None*
Villalta et al. (53)	Hepatitis C virus	Positive
Ruggeri et al. (54)	Hepatitis C virus	Positive
Sarmiento et al. (55)	Enterovirus, EBV, CMV, Hepatitis C virus	Positive
Tjernberg and Ludvigsson (56)	RSV	Positive
Abid et al. (57)	Hepatitis B virus	Positive
Tarish et al. (58)	Adenovirus	None
Alaedini et al. (59)	Borrelia	None
Bouziat et al. (60)	Reovirus	Positive

CD, celiac disease; CDA, celiac disease autoimmunity; EBV, Epstein Barr virus; CMV, cytomegalovirus; HSV, herpes simplex virus; RSV, respiratory syncytial virus.

[#] *Case reports were excluded.*

* *Between these infection during pregnancy and CD development in the offspring.*

Type of Delivery

The mode of delivery (vaginal or cesarean section [C-section]) has a strong influence on shaping the initial gut microbiota

TABLE 7 | Some of the most relevant studies on type of delivery and the risk for celiac disease.

First author, year, study	Conclusion
Koletzko et al. (73), TEDDY	No association with CDA or CD
Dydensborg Sander et al. (74), ETICS	No association with CD
Lionetti et al. (72), CELIPREV	No association with CD
Kristensen and Henriksen (68)	Positive association between emergency CS and CD
Emilsson et al. (70), MoBa	No association between CS and CD
Sevelsted et al. (71)	No association with CD
Marild et al. (69)	Positive association with CD
Decker et al. (31)	Positive association with CD
Roberts et al. (32)	Negative association between CS and CD

CD, celiac disease; CDA, celiac disease autoimmunity; CS, cesarean section.

composition. It has been hypothesized that infants born by C-section acquire different bacterial communities compared to vaginally delivered infants (64), which may influence the short- and long-term immune responses to environmental factors, thereby predisposing to autoimmunity (65). Also, the type of C-section, emergency vs. elective, has been hypothesized as a different possible influencing factor, since the cord blood immune cell phenotypes are affected by stress during vaginal delivery and this does not happen by elective C-section (66). In addition, infants born vaginally and during emergency C-section are colonized at first by fecal and vaginal bacteria of the mother, whereas infants born through elective cesarean delivery are exposed initially to bacteria originating from the hospital environment and healthcare workers. Infants born by cesarean delivery are characterized by a more slowly diversifying microbiota, with a substantial absence of Bifidobacteria species and Bacteroides and the presence of facultative anaerobes, such as Clostridium species. These differences might influence the development of the mucosal immune system, the establishment of a stable intestinal host-microbial homeostasis, as well as the mucosal barrier function and ultimately contribute to the risk of acquiring immune-mediated diseases, such as CD, later in life (67).

Some studies have identified C-section as a risk factor for the development of CD (68, 69). However, more recent prospective studies have found no association (70–73) (**Table 7**). Recently, a large, observational, register-based, cohort study investigated the association between the type of delivery and the risk of developing CD in two independent population cohorts (Denmark, birth cohort 1995–2010 and Norway, birth cohort 2004–2012) (74). A total of 3,314 children were diagnosed with CD. C-sections were performed in 286,640 children, and the mode of delivery was not associated with an increased risk of diagnosed CD.

In the above-mentioned Danish cohort, the association between elective C-section and diagnosed CD was positive and reached borderline statistical significance after adjusting for year of birth, sex, maternal age, education, parity, gestational age, and weight for gestational age (OR: 1.20; 95% CI: 1.00–1.43). However, this finding was not replicated in the corresponding Norwegian cohort (OR: 0.96; 95% CI: 0.79–1.17) (74). Analysis of the data from the Swedish Medical Birth Register between 1973 and 2008, comparing cases with villous atrophy with age- and sex-matched controls from the general population, found a weak association between an elective C-section and CD in offspring (adjusted odds ratio [OR] = 1.15), but no increased risk for CD diagnoses after an emergency (adjusted OR = 1.02) or any C-section (adjusted OR = 1.06) (69). Data from a population- and national register-based cohort including all children born in Denmark from January 1997 to December 2012 showed the opposite: children delivered by emergency C-section were at an increased risk for CD (adjusted OR = 1.22), whereas children delivered by elective C-section were not (adjusted OR = 0.69) (68). Thus, despite the plausible hypothesis that mode of delivery affects risk of CD, the current literature showed no association between the type of delivery and the risk of CD (**Table 7**).

Antibiotics

The ETIC study found no evidence of increased CD risk with antibiotic use in the first 6 months of life (61). However, other 2 retrospective studies have shown a positive association between antibiotic use and CD risk (75, 76). A recent analysis of the TEDDY study showed that cumulative exposure to β-lactam or macrolide antibiotics, up to 6 months, during the first or second year of life and within 6 months before the seroconversion period, was not associated with CDA. Also, maternal use of antibiotics during pregnancy was also evaluated as a risk factor and did not significantly contribute to CDA risk in this study. In conclusion, the role of antibiotics in the development of CD is a topic that remains unclear and requires more research.

Microbiota

CD development has also been linked to alterations in the human gut microbiome, which is necessary for proper development of the immune system and establishment of oral tolerance in early life (65). The contributing role of perturbations in the gut microbiota, and of specific enteric bacteria, to gluten-induced immunopathology has been shown in animal models (77). PROFICEL, a prospective study of 164 healthy Spanish newborns with a first-degree relative with CD and HLA-DQ2 and/or DQ8 positivity, reported an association between the HLA-DQ genotype and the intestinal microbiota composition. In this study, the HLA-DQ2/8 genotype and the type of feeding (breastfeeding or formula) were shown to influence, in conjunction, the composition of the intestinal microbiota (78). The high-risk genotype for developing CD (HLA-DQ2, including homozygous HLA-DQ2.5 or heterozygous DQ2.5/DQ2.2 and DQ2.2/DQ7.5) was associated with reduced numbers of Bifidobacterium, specifically of the species *B. Longum,* compared to the rest of the lower-risk genotypes (79). Also, other studies have found similar results; the duodenal and fecal microbiota of CD patients is unbalanced, with decreased numbers of anti-inflammatory bacteria, such as Bifidobacterium spp. and increased numbers of Bacteroides spp., which are only partially normalized after a long-term gluten free diet (GFD) (80–82). In a double-blind, randomized, placebo-controlled, interventional trial performed in children with newly diagnosed

CD, children were randomized to receive *Bifidobacterium longum* or placebo in conjunction with a GFD (83). A decrease in both the numbers of the Bacteroides fragilis group and the fecal secretory IgA concentration was found, which might further confirm the role of microbiota in the pathogenesis of CD. But, so far, studies have failed to find a distinct microbiota profile in patients with CD.

A sub-study of the PROFICEL project, including 10 CD cases and 10 matched controls, suggests altered early proportions of Firmicutes and members of the Actinobacteria phylum (*B. Longum*) in children who later progressed to CD (84). Hopefully, the results of the Celiac Disease Genomic, Environmental, Microbiome, and Metabolomic (CDGEMM) study, a multicenter, longitudinal study of infants at risk for CD, will provide an answer to the question regarding the role of the gut microbiome and the risk of CD (85). CDGEMM aims to enroll 500 infants aged 0–6 months with a first-degree family member with CD. Health status, anthropometrics, nutritional information, household and environmental information, and blood and stool samples are being collected regularly to understand the role of the gut microbiome as an additional factor that may play a key role in early steps involved in the development of autoimmune disease (85).

In conclusion, in the field of primary prevention, infant feeding practices have been explored by interventional studies with long-term follow up, but have shown no protection for risk of CD. Other possible influences on the development of CD, especially the role of infections and the gut microbiome, need further research.

Text box

Summary of evidence of effectiveness of possible primary prevention strategies for celiac disease	Conclusion
Infant feeding	
Breastfeeding	No effect
Breastfeeding at the time of gluten introduction	No effect
Timing of gluten introduction	No effect
Amount of gluten at the time of gluten introduction	Unclear
(Intestinal) infections	Unclear
Type of delivery	No effect
Antibiotics	Unclear
Microbiota	Unknown

SECONDARY PREVENTION

Case Finding

Secondary prevention focuses on early detection and treatment (**Table 8**). Active case finding refers to the liberal diagnostic testing of subjects with CD-associated symptoms. In the general population, this approach has led to the early diagnosis of many patients, resulting in significant health improvement after treatment, good compliance with the GFD, and good CD-related quality of life (86, 87); unfortunately, however, it does not counter the entire under-diagnosis of CD (88, 89). Only a small proportion of the undiagnosed patients are detected with this strategy, since ∼50% of the children in screening-detected studies have symptoms at the time of diagnosis (15, 16, 90).

TABLE 8 | Secondary prevention strategies for celiac disease.

Case finding
Screening in high-risk groups
First-degree relatives of CD patients
Type 1 diabetes mellitus
Autoimmune thyroid disease
Autoimmune liver disease
Syndrome: Down, Turner, Williams
IgA deficiency
Mass screening

CD, celiac disease.

Screening for Celiac Disease in High-risk Groups

Because of the high prevalence of CD among these groups, evidence-based guidelines recommend screening for early detection of the disease (7) (**Table 8**). A plethora of studies are available on 2 of the populations who belong to these high-risk groups, namely first-degree relative of patients with CD and children with type 1 diabetes mellitus (T1DM).

First-degree Relatives of CD Patients

Many studies have demonstrated that first-degree relatives (FDRs) of celiac patients have a higher risk of developing CD than the general population, with a prevalence ranging from 1.6 to 38% (91). Based on a systematic review and meta-analysis, Sing et al. (91) reported that the pooled prevalence of CD was 7.5% in 10,252 FDRs (91). The risk of developing CD among FDRs is influenced by gender and HLA haplotype (15, 16). CD occurs more often in girls (female: male ratio of 2–3:1), and HLA-DQ2 homozygous children have a significantly higher risk of developing CD than HLADQ2 heterozygous children (14.9 vs. 3.9%, respectively, at the age of 3 years) (15).

Children With Conditions/Diseases Associated With CD

The prevalence of CD in patients with T1DM has been reported by most studies as ranging between 4 and 10% (92). Many children with T1DM and CD are asymptomatic or at least symptoms of CD have not been observed. In these cases, CD may only be detected by serologic screening. However, it has been shown that strict adherence to a GFD was <30% in children with both CD and T1DM, compared to 81% among patients with CD only (93). Maintaining a strict GFD in addition to a diabetic diet requires additional time, effort, and expense. Evidence is inconclusive as to whether the benefits of screening and potentially treating asymptomatic individuals outweigh the harms of managing a population already burdened with a serious illness. The Celiac Disease and Diabetes-Dietary Intervention and Evaluation Trial (CD-DIET) (ClinicalTrials.gov Identifier: NCT01566110) involves screening of children and adults with T1DM for asymptomatic CD, followed by randomization to a

GFD or no-GFD group, to assess outcomes (including diabetes control, bone mineral density, and health-related quality of life) over 1 year to clarify effects of screening and treating asymptomatic CD in this population with a GFD (94).

Mass Screening

Screening the general population, also called mass screening, would theoretically be the best form of secondary prevention since it could potentially detect all cases of CD, including those in asymptomatic patients as well as those in patients who lack symptoms. Results from most screening studies performed in the general population suggest that symptoms are not reliable predictors of CD (15, 95, 96), reinforcing the place of mass screening as the best strategy for secondary prevention of CD enabling early treatment to reduce the burden of morbidity and mortality associated with untreated CD (97, 98).

However, mass screening for CD is still debated, partly because evidence has been lacking on the accuracy of diagnostic tests and on the health benefits after diagnosis and treatment of asymptomatic detected patients. This uncertainty also affects the cost benefit of mass screening, which is needed for implementation of mass screening for CD (99, 100). Most studies on the diagnostic accuracy of diagnostic tests for CD have been conducted in symptomatic patients (101, 102). Because the positive predictive value declines when the test is used in settings with a low pre-test prevalence, such as the general population, the sensitivity and specificity of these tests are lower in the setting of mass screening.

Recently, a prospective study performed in Rotterdam has shown a positive predictive value of 81% for CD in the general pediatric population (96). These authors also showed that undiagnosed CD is associated with a lower body mass index compared to controls at the age of 9 years (96) and associated with fetal growth restriction and lower birth and placental weight during pregnancy (8). Additional information about the importance and effectiveness of screening comes from a population-based-screening study performed in Sweden; this study showed that at 10 years of age, children with CD detected by screening already had reduced bone mineral density in the total body and spine compared with age-matched controls. These differences were not found in children with CD on a GFD from 3 years of age, indicating that children with screening-detected CD benefit from early diagnosis and treatment (103).

The data on the benefits and harms of screening are limited. Only one randomized trial evaluated the effectiveness of GFD vs. no GFD in apparently asymptomatic adults with screen-detected CD and found that initiation of a GFD in screen-detected adults with unrecognized symptoms was associated with improved gastrointestinal symptoms (104).

Other traditional reason against mass screening are that adherence to the GFD in minimally or asymptomatic patients would be lower than in symptomatic patients and that the quality of life is decreased in screening-detected CD patients following a GFD. However, 10 years of follow up among Dutch children and the results of a sub-study from the ETICS project showed similar adherence rates to the GFD in screening-detected children compared with clinically detected children (105, 106). No significant differences in health-related quality of life (HRQoL) were observed between screening-detected and symptom-detected adult patients (107, 108). Furthermore, a systematic review and meta-analysis on dietary adherence and HRQoL in adult patients with CD detected by screening showed a significantly lower HRQoL after 1 year of treatment with a GFD in symptom-detected patients compared to screening-detected patients (109). Despite the aforementioned literature that is positive about screening of the general population, the current literature recommending mass screening is limited.

TERTIARY PREVENTION

Gluten-free Diet (GFD)

Tertiary prevention focuses on reducing the impact of existing disease by improved treatment (**Table 9**). One of these strategies involves optimizing adherence to the GFD. Complete removal of gluten from the diet is a challenge, as gluten is present in a wide variety of foods. However, since the introduction of allergen labeling in the European Union (EU) in 2005, gluten cannot be hidden in products. The amount of gluten capable of initiating an antigenic reaction has been estimated to be >20 mg/kg (or parts per million = ppm) of gluten, and contamination below 20 ppm is considered safe over a wide range of foods in daily consumption.

Improving Monitoring of and Adherence to the GFD

Dietitian

Due to the complexity of the GFD, it is essential that newly diagnosed patients be referred to a dietitian with expertise in CD. A delay in referral, or no referral at all, increases the likelihood of the patient obtaining inaccurate information from the Internet, health food stores, alternative health practitioners, family, friends, and other sources, often resulting in confusion, frustration, and insufficient knowledge regarding CD and the GFD (110).

Gluten-containing cereals, such as wheat, barley, and rye, are important sources of dietary iron, calcium, folate, and vitamin B12. As the treatment of CD with a GFD can lead to nutritional deficiencies, the support of a dietitian is necessary to avoid these deficiencies. Also, consultation with someone with knowledge in

TABLE 9 | Tertiary prevention strategies for celiac disease.

Strategy	Successful
Optimal adherence to the GFD	Yes
Treatment options for CD other than a GFD	
Larazotide acetate	Unclear
Endopeptidases	
Latiglutenase (ALV003)	Unclear
Aspergillus niger prolyl endoprotease (AN-PEP)	Unclear
Desensitization therapy (therapeutic vaccine)	Unknown

CD, celiac disease; GFD, gluten-free diet.

the field of replacement (gluten-free) products, such as amaranth, buckwheat, quinoa, sorghum and teff, is of great importance and could improve intakes of protein, iron, calcium, and fiber by patients with CD (111).

Validated Food Questionnaires

A dietary interview to assess compliance with the GFD is the best way to detect errors in GFD adherences among children and young adults, but it is timue-consuming (20–30 min per patient) and requires expert personnel. Several short questionnaires have been developed to measure GFD adherence in order to save time, and while some are not sensitive enough, others are useful in assessing compliance to the diet (112). With the increasing use of self-assessment and alternative follow-up methods for CD patients, including electronic patient records and E-health tolls, completing questionnaires before or during a medical consultation should be easily implemented in the healthcare of children and young adults with CD (113).

Measurement of Gluten Immunogenic Peptides (GIPs)

Available methods to assess GFD compliance are time-consuming and are also insufficiently sensitive to detect occasional dietary transgressions that may cause gut mucosal damage. Determination of serum TG2A is usually used during the follow-up of a patient on a GFD, as this marker improves with gluten elimination (114). However, it has been reported that even while following a GFD, children and women with CD have a much higher prevalence of gastrointestinal symptoms than controls, and they also use healthcare services more often (115). As mucosal damage may still persist without TG2A, antibody testing may be negative in patients with only partial adherence to the GFD (116). Therefore, it is necessary to have a non-invasive biomarker to monitor compliance with the GFD. Certain GIPs are resistant to gastrointestinal digestion and can interact with the immune system of patients with CD to trigger an autoimmune response against transglutaminase and other antigens. A proportional fraction of the GIPs absorbed in the gastrointestinal tract make it to the circulation and are excreted in urine. GIPs are detectable in concentrated urines and may be useful in clinical practice as a monitoring tool to follow-up compliance with the GFD. GIPs are detected in urine samples 6–48 h after gluten intake (>25 mg) and remained detectable for 1–2 days (117).

Treatment Options for CD Other Than the GFD

Several other treatments aimed at different pathogenic targets of CD have been studied in recent years: modification of gluten to produce non-immunogenic gluten, endoluminal therapies to degrade gluten in the intestinal lumen, increasing gluten tolerance, modulation of intestinal permeability, and regulation of the adaptive immune response. However, not all of these therapies have been tested in clinical trials (yet). The most advanced studies are devoted to larazotide acetate and prolyl-endopeptidases degrading toxic gluten peptides and to therapeutic vaccination.

Larazotide Acetate

Patients with active CD have increased intestinal permeability. Zonulin, a modulator of epithelial tight junctions, is overexpressed in these patients. Release of zonulin in response to binding between gliadin peptides and a specific chemokine receptor (CXCR3) results in a measurable reduction in the usual intestinal barrier and allows enhanced passage of gliadin. This mechanism has been the target of advanced research that led to the development of larazotide acetate (AT-1001), an octapeptide that inhibits gliadin-induced intestinal permeability. Several phase I and II clinical trials have confirmed the safety of this agent and suggest a potential beneficial effect of larazotide (118, 119). Additionally, patients who were treated with larazotide acetate had significantly fewer symptoms (patient reported Celiac Disease Gastrointestinal System Rating Score) compared with those taking a placebo (120–122). A dose-response effect was not seen, with the most benefit encountered at the lowest (0.5 mg) of 4 dosages administered (121); however, this study did not measure histologic endpoints, and larazotide had no significant effect on serologic levels of specific CD antibodies as TG2A.

Endopeptidases

The gluten peptides, which are responsible for inducing the immunological response in CD patients, are rich in proline and are highly resistant to enzymatic proteolysis within the digestive tract. For many years, there have been studies conducted to investigate the effectiveness of orally administered prolyl oligo-peptidases in the degradation of toxic gliadin peptides before they reach the mucosa of the small intestine.

Latiglutenase (ALV003, Alvine Pharmaceuticals, San Carlos, CA, USA) is an orally administered mixture of 2 recombinant gluten-specific proteases—a cysteine protease (EP-B2) and a prolyl endopeptidase (PEP)—which have been shown *in vitro* to degrade gluten (123). Both endopeptidases are active and stable at gastric pH (124). In a Phase 2 study with ALV003, adults with biopsy-proven CD were randomly assigned to groups receiving ALV003 or placebo, together with a daily 2 g gluten challenge. Upper endoscopy was performed at baseline and after the gluten challenge. Primary endpoint included the villus height to crypt depth ratio and $CD3^+$ intra-epithelial lymphocytes (IEL) density. Serologic markers and symptoms were also assessed. In the ALV003 group, there were no changes in histological measures, while in the placebo group, evidence of mucosal injury was shown after gluten challenge. In contrast, no differences were seen in symptoms and serologic markers of CD in both groups. In a phase 2 study involving patients with symptomatic CD and histologic evidence of significant duodenal mucosal injury, ALV003 did not improve histologic and symptom scores when compared with placebo (125). However, a subgroup-analysis of the study showed a statistically significant, dose-dependent reduction in the severity and frequency of symptoms in seropositive but not in seronegative patients (126).

Aspergillus niger prolyl endoprotease (AN-PEP; DSM, Heerlen, The Netherlands) is also an endopeptidase, isolated from the fungus Aspergillus niger. The enzyme is active between a pH of 2 and 8, with an optimum activity at pH 4–5, thus, in

the stomach and small intestine (127). In a randomized, placebo-controlled, crossover study, 18 self-reported gluten-sensitive subjects consumed a porridge containing 0.5 g gluten together with two tablets containing either a high or low dose of AN-PEP or placebo. Gastric and duodenal contents were sampled over 180 min. The primary outcome was defined as the efficacy of the high dose of AN-PEP compared with placebo in degrading at least 50% of gluten, based on the amount of gluten detected in the duodenum. The researchers concluded that the AN-PEP enzyme is effective in degrading small amounts of gluten as part of a complex meal in the stomach, but it is not intended to replace a GFD in patients with gluten-related disease (128). In a Phase 2a double-blinded, placebo-controlled, randomized trial, 16 CD patients on a GFD, who were in serological and histopathological clinical remission, were administered AN-PEP or a placebo with gluten-containing toast (∼7 g/day gluten). The mean score for the gastrointestinal subcategory of the CD quality (CDQ) was relatively high throughout the study, indicating that AN-PEP was well-tolerated. In the efficacy phase, the CDQ scores of patients consuming gluten with placebo or gluten with AN-PEP did not significantly deteriorate and, moreover, no differences between the groups were observed.

Larazotide and PEPs will not become an alternative to the GFD and their potential role as therapeutic agents for CD remain unclear.

Desensitization Therapy (Therapeutic Vaccine)

NexVax2 from ImmusanT is a desensitizing vaccine that uses three dominant gluten peptides administered subcutaneously to induce an immune response in CD patients who carry the immune recognition gene HLA-DQ2.5, which accounts for disease in 80–90% of patients. The aim of the vaccine is to use peptide-based immunotherapy to shift the T-cell response from pro-inflammatory to regulatory, in order to restore immune tolerance to gluten. Phase 1b clinical trials of this vaccine have recently been completed supporting the safety, tolerability and relevant bioactivity of Nexvax2 (129).

CONCLUSIONS

- Celiac disease is a common autoimmune disorder induced by ingestion of gluten in genetically susceptible individuals.
- Only a minority of those who are at genetic risk develop the disease.
- The incidence of CD has increased over the last half-century, resulting in rising interest in identifying risk factors for CD to enable prevention.
- Environmental and/or lifestyle factors play a causal role in the development of CD.
- For primary prevention (i.e., interventions before CD occurs), early feeding practices seem to have no impact on the risk of developing CD during childhood. Other environmental influences have been investigated as potential risk factors; however, they have not yet led to primary prevention strategies.
- Secondary prevention is possible through early diagnosis and treatment; however, it will not identify all CD patients as long as mass screening has not been introduced.
- As a gluten-free diet is a major challenge, tertiary prevention strategies are under evaluation; however, none of these measures are currently recommended as treatment.

AUTHOR CONTRIBUTIONS

CM: Corresponding author, primary responsibility for communication with the journal during the manuscript submission, drafting the work, final approval of the version to be published, agreement to be accountable for all aspects of the work. RS: Critical revision of the article, final approval of the version to be published, agreement to be accountable for all aspects of the work. HS: Critical revision of the article, final approval of the version to be published, agreement to be accountable for all aspects of the work. LM: Drafting the work, supervising corresponding author, final approval of the version to be published, agreement to be accountable for all aspects of the work.

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REVIEW
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Microbial Transglutaminase Is Immunogenic and Potentially Pathogenic in Pediatric Celiac Disease

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The enzyme microbial transglutaminase is heavily used in the food processing industries to ameliorate food qualities and elongate the products' shelf life. As a protein's glue, it cross-links gliadin peptides, creating neo-complexes that are immunogenic and potentially pathogenic to celiac disease communities. Even lacking sequence identity, it imitates functionally the endogenous tissue transglutaminase, known to be the autoantigen of celiac disease and representing an undisputable key player in celiac disease initiation and progress. The present review expend on the enzyme characteristics, exogenous intestinal sources, its cross-linking avidity to gluten or gliadin, turning naïve protein to immunogenic ones. Several observation on microbial transglutaminase cross linked complexes immunogenicity in celiac patients are reviewed and its pathogenicity is summarized. Warnings on its potential risks for the gluten dependent conditions are highlighted. When substantiated, it might represent a new environmental factor of celiac disease genesis. It is hoped that the presented knowledge will encourage further research to explore the mechanism and the pathogenic pathways taken by the gliadin cross linked enzyme in driving celiac disease.

Keywords: celiac disease, microbiome, food additive, gluten, cross linking, microbial transglutaminase

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INTRODUCTION

Celiac disease (CD) is an autoimmune disease, thus dependent on genetic background and on environmental factors. The list of the environmental components that might impact CD initiation or development is continuously expanding, but the precipitating events causing CD remain enigmatic. Except for the prolamins, none of them reached cause and effect relationship. The introduction is divided to 4 sub-heading in order to set the stage for microbial transglutaminase (mTg)-CD cross-talks.

Environmental Factors Associated With Celiac Disease

A plethora of environmental components were suggested to influence CD, spanning infections, food, drugs, vaccination, toxins and metals, abdominal or gynecological surgery, level of hygiene, socio-economic status, life style stress and processed food additives (1–5). Microbes like *Helicobacter pylori*, *Campylobacter jejuni*, pneumococcus, bacteroides species and tuberculosis were associated. In the virus's domain: CMV, HBV, HCV, Rotavirus, Adenovirus, Reovirus, and enteroviruses were implicated. To complicate the topic, same bacteria and viruses might play a

protective role in CD (4). Pharmacological factors are early antibiotic therapy and proton pump inhibitors. Influenza and HPV vaccines, Heavy metals, Aflatoxin, smoking, alcohol, Cesarean section and abdominal surgery, Western higher hygiene and higher socioeconomic level, stressful life and Westernization of the diet, microbiome dysbalance characterized by an abundance of Proteobacteria and a decrease in Lactobacillus were associated with CD development or increased incidence (6, 7). Most recently the processed food additive were suggested to influence the intestinal microbiome and to increase intestinal permeability, thus contributing to luminal eco-events that drive autoimmunity (8). One of them is the mTg, the topic of the present review. In 2015, the hypothesis that mTg might play a role in CD genesis was forwarded and since then more studies were conducted to substantiate this hypothesis (9).

MTg: Characteristics, Functions, Food Applications

Prokaryotic mTg is a part of the global transglutaminases family (10), isolated and characterized from multiple bacterial strains, *Streptomyces mobaraense* being the first one (11, 12). The *S. mobaraense* mTg harbors 331 amino acids, its molecular weight is 37.9 kDa. Notably, list of additional microbes that secrete the enzyme and their enzymatic yield capacities is continuously expanding (13), a recent one is a novel Transglutaminase from *Streptococcus suis* (14).

Being a member of the Tg family, mTg catalyzes the formation of an isopeptide bond, cross-linking an amine group (containing the acyl acceptor lysine) and the acyl group (containing the acyl donor glutamine). As gluten is abundant in glutamine (∼30%) and contains lysine (<2%) (15), representing an acyl donor and acceptor, respectively, it is an ideal substrate for post-translational modification of gluten, by transamidation, or deamidation. Based on its peptide modifying capacity it imitates functionally the endogenous tTg, the ultimate autoantigen in CD (9, 13, 16, 17). It should be emphasized that bonds formed by the mTg resist proteolytic degradation, it exhibits a higher reaction rate, substrate specificity, higher transamidation/ deamidation ratio resulting in improved cross-linking capacity. Due to its broad enzymatic activity, it is heavily used by the food processing industries (8, 9, 12, 18–23). In fact, the enzyme is consumed by most of the processed food industries, spanning the meat, dairy, sea food and fish, surimi, casein and gelatin, myosin and actin, confection, and convenience ones and many more (8, 9, 12, 18–22). The net % increase per year of enzyme usage in the processed food industries is estimated to 21.9%, mTg being a major one (8). In the food processed industries, mTg improves gelation and changes emulsification, foaming, viscosity and water-holding capacity. It is considered as the "glue of proteins" and polymerization agent, thus improving food palatability, texture and life time on the supermarkets' shelves.

Zooming on CD and the increased affinity of the mTg to gluten, the enzyme in increasingly used in the bakery industries (8, 9), where it lowers the products calories, improves texture, elasticity and dough characteristics (9, 12, 24).

Summarizing the literature, an estimated daily intake of mTg used in the processed food products can range up to 15 mg where every kg of mTg processed product contains around 50–100 mg of mTg (18, 25, 26). Finally, a direct positive correlation is found between the increased annual usage of industrial enzymes added to processed bakery products and the increase in CD frequency, in the last four decades (7, 13). It should be stressed that we are dealing with an associative correlation and no causality was determined.

MTg Is Structurally Different but Functionally Imitates the tTg

In contrast to endogenous human tTg, the microbial one is a calcium and nucleotide independent enzyme. It consists of a single domain, compared to the four domains of the tTg and has around half the molecular weight. MTg operates at a larger range of pH, buffers, and temperatures and has a much larger list of substrates. So, in opposition to the tTg, mTg less substrate specific. Those features are advantageous for many industrial applications and it is clear why the industries preferred mTg over its member, tTg. MTg lacks sequence homology to tTg, but, due to their active site performances, mTg has substantial functional similarity (9, 13, 18). Not surprisingly, the two enzymes can transamidate or deamidate proteins, based on the enzymatic reaction's conditions (23).

The Luminal Intestinal Sources of mTg

MTg can originate from intra luminal sources and from external sources. The luminal enteric sources come from the gut microbiome. Our lumen is over-crowded by bacteria but also by archaea, viruses and protozoa, reaching roughly 10^{14-16}, and the ratio of microbes/ host cells ratio averages 1:1. Its dimensions, composition, diversity, mobile products and activities have led to its description as a "superorganism" (27). A component of the microbial Metabolome represented as a mobilome is the secreted mTg. It appears that by using sequence search programs, hundreds to thousands of mTgs-encoding bacteria can be detected, the majority belong to the Firmicutes phylum (13, 16). As a survival factor mTgs are secreted by the microbes in order to survive their overpopulated luminal compartment. When intestinal fluid was analyzed, the fluid was positive for transglutaminase activity, however, the authors did not distinguish between the endogenous tTg and the bacterial one, but to our knowledge, no tTg activity was reported in the duodenal lumen, so far, in contrast to the intestinal mucosa and the lamina propria (28).

The extra intestinal sources of mTg are much wider. Mtg as a processed food additive was extensively discussed above (8, 9, 12, 13, 16, 18–22). Belonging to the prokaryotes, the probiotics represent a reservoir of mTg, representing an active cargo that affects luminal events. It is well-known that microbes can transfer virulent factors to their mates by horizontal gene transfer (29). Major concern on the transfer of virulent genes, like antibiotic resistant genes, via the nutrients and food chain exists and many of those deleterious genes are carried by probiotics (30–32).

Abbreviations: CD, celiac disease; mTg, microbial transglutaminase; tTg, tissue transglutaminase.

The virulent genes were described to end up in the human gastrointestinal tract microbiota (33). The factors causing the transformation from benign inhabitant of the gut microbiome or ingested probiotics to virulent pathogen are not clear, but a combination of horizontal gene exchange of virulence factors and differential transcription of endogenous genes are clearly involved (34).

An additional enzymatic cargo of mTg delivered to the enteric lumen comes from the pathobionts. In addition to their capacity to post-translate and modify protein and breach tight-junction integrity (8, 13, 16) they add pathogenic burden, represented by the mTg, as was recently described (14)**.** The environmental pathogenic microbes can exchange mobile elements with the luminal inhabitants. Surface water, sewage treatment plant effluents, soils, animal wastes, contaminated biofilms and urban rivers are increasingly reported, thus threatening global human health (35–37). Not surprising, commonly consumed plants and vegetables contain transglutaminase. Even if their sequence homology to mTg is not high, they are capably to cross-link peptides, including gluten. They were even implicated as a possible player in CD pathogenesis, starting the process in the intestinal lumen (38). MTg carrying examples are apple, soybean, bean sprouts, fodder beet, rosemary leaves, Jerusalem artichoke, spinach leaves and green peas, routinely consumed/ingested plants, fruits and vegetables, all have transglutaminase activities (38). Polyols, heavily used in the processed food industry in protein-based coating, biofilm formation, gelatination of products and bio-packaging, were shown to improve thermal stability and half-life of the mTg, thus potentiating the enzyme cross linking capacity (39).

A totally new topic of potential Tg delivery to the gut lumen is coming from the microbiota yeast's domain. Significantly higher fecal counts of candida and saccharomyces species were identified in CD patients (40) and CD patients have higher frequency and levels of anti-*Saccharomyces cerevisiae* antibodies (41). Both, *Candida albicans* and *Saccharomyces cerevisiae* synthesize the Tg enzyme (42, 43) to the point that *Candida albicans* was suggested to trigger CD (44, 45).

Summarizing the present paragraph, a plethora of extra intestinal and luminal sources of mTg exist that potentially can interact with gluten containing compounds. This enzymatic cargo can cross link those glutamine rich peptides, resulting in post-translated, modified immunogenic epitopes, potentially driving CD autoimmunogenesis (9, 13, 16). After setting the introductory stage, the following part will expend on the mTg cross linked gliadin complex immunogenicity and potential pathogenicity in CD.

GLIADIN DOCKED MTG COMPLEXES ARE IMMUNOGENIC IN PEDIATRIC CD

When mTg transamidates gliadin peptides a neo cross-linked complex is created, where the physico-chemical, electrical and three dimensional features are changed (46). This is a typical enzymatic post-translational modification of gliadin, resulting in a non-naïve, non-tolerogenic and potentially immunogenic protein (13, 16). To investigate its immunogenicity, CD associated antibodies, including the anti-neo-epitope mTg were investigated, back to back, in a pediatric CD population, compared to controls. The neo-epitope mTg IgG had a sensitivity of 94.9% and specificity of 93.9% and reflected the patient's intestinal pathology (46). Performing competition assays between the isolated antigens (gliadin, tTg and deamidated gliadin peptide) and the gliadin tTg/mTg neo complexes, mTg and tTg-neo epitopes display comparable immunopotent epitopes (46).

After describing the mTg-gliadin complex antigenicity in CD, the stage is ready to evaluate mTg potential pathogenicity in driving CD.

PATHOGENIC ASPECTS OF MTG

Several aspects of the mTg hint at its potential pathogenicity and suggest involvement in CD initiation/progression. Sharing functional aspects with the autoantigen and the mucosal step limiting phase of the tTg enzyme in CD development puts mTg as a primary candidate as a partner for CD development. Following are several observations that support mTg involvement in the CD autoimmunogenesis.

MTg Suppresses Intestinal Luminal Protective Barriers

The human intestine possess multiple protective mechanisms to keep the microbes at bay. Several of those mechanisms are contradicted by the mTg, when bugs are fighting to survive inside us:

a. The highly physiological important isopeptide bonds created by the mTg are resistant to any known human enzyme, thus escaping the enzymatic hydrolysis, reducing or chaotrophic agents and detergents. Those mTg formed protective structures improve the microorganism's survival in the enteric hostile compartment. Even bile acids, antimicrobial peptides and immunoglobulins are ineffective facing those structures (13, 47, 48).
b. mTg was found to suppress human immunity by its anti-phagocytic property, thus suppressing a major immune protective system (14, 49, 50).

MTg Effects on Epithelial Gliadin Uptake and Transportation

a. MTg may enhances gliadin uptake through human intestinal cell-line, as was demonstrated for tTg. TTg was shown to facilitate apical-basal passage of gliadin peptides, cooperating with the apical transferrin receptor and secretory IgA (9, 13, 51). Imitating tTg functions, mTg potentially can facilitate this epithelial gliadin uptake pathway, thus enhancing CD.
b. MTg and gliadin share comparable trans-enterocytic transport pathway. A major indispensable step in the pathophysiology of CD is the transcytosis of gliadin peptide to the sub-epithelial space to be deamidated\ transamidated by the

tTg. It appears that mTg and gliadin peptides use this port of entry and pathway. Most Lately, Stricker et al. (52) placed tagged mTg and gliadin to the enterocyte upper membrane of CD and control human intestinal specimen or human cell line. A simultaneous uptake of mTg and gliadin into the endoplasmic reticulum of the RACE-cells and co-localization of the mTg and gliadin at the basolateral membrane and the lamina propria of CD duodenal samples were detected. If their results are substantiated it might reinforce the potential pathogenic role of the exogenous mTg or mTg-gliadin complex in CD evolution, when the complexes are exposed to the local immune systems. In this case, one can envision that the sub-epithelial deposits of tTg-gliadin, described as an early histological finding in CD intestinal samples, contain mTg-gliadin complexes.

MTg can Potentially Enhance Gliadin Induced Intestinal Permeability

Several mechanisms can explain how mTg functions and products can increase enteric permeability:

a. Protecting intestinal dysbiota and pathobionts, the mTg can enhance their luminal survival. Intestinal infections are important breachers of tight junction functional integrity (53).
b. Gliadin is an ideal substrate for mTg cross-linking. Being a major disruptor of intestinal permeability (54), its cross-linked complexes might further augment gut permeability.
c. Some tight junction proteins and cytoskeleton elements like actin, e-cadherin or adherens junctions can be modified by Tgs (55, 56). By cross-linking those proteins the mTg can disrupt tight junction functionality (9).

Additional Facts Related to Potential mTg Pathogenicity in CD

In order that the industrial processed food additive will impact human health, it has to be present in the consumed products. Notably, when 60 meat and meat products from the supermarket shelves were checked by two sensitive (around 25 mg pure enzyme in 1 kg of product) analytical methods, many contained the enzyme mTg (57). A literature survey disclosed that around 50–100 mg of mTg is used to process 1 kg of food product and the average intake of mTg can amount to 15 mg per day (12, 18, 25, 26). Although not causative, epidemiological data show an associative correlation between the surges of CD incidence and the consumption of enzymes in the bakeries, mTg being a major one (8, 9). Finally, wheat or gluten containing products enzymatically treated by mTg were shown to be immunogenic, inducing antibodies when consumed by humans (9, 46, 58–67).

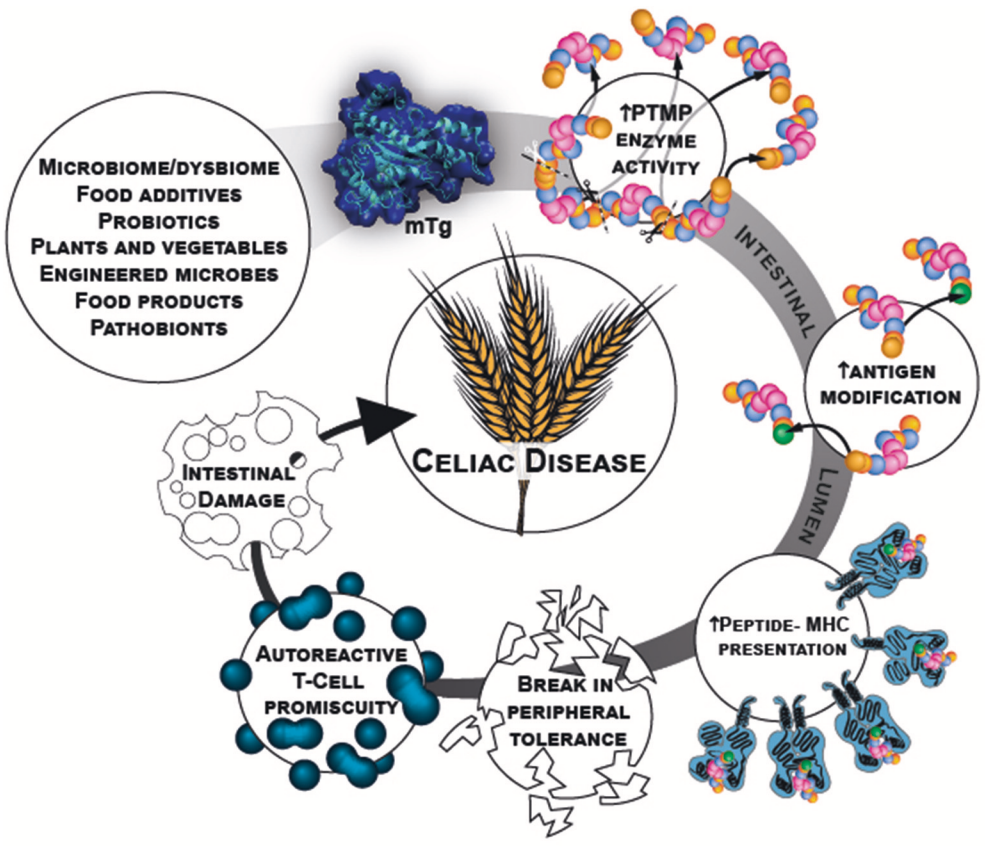

FIGURE 1 | mTg sources and chain of events that potentially initiate gluten break of tolerance resulting in CD.

ACADEMICAL AND AUTHORITATIVE WARNINGS ON THE POTENTIAL HARMFUL FUNCTIONS OF MTG USAGE BY THE PROCESSED FOOD INDUSTRIES

The mTg is not labeled, since it is considered as a processing aid, thus escaping the definition of a food additive. Due to the potential detrimental public health aspects, several scientists and organizations issue warnings, trying to increase the awareness of the regulatory authorities, academical communities and the general public on the subject. Following are some citations: "The usage of transglutaminase as a food additive is permitted in some countries. However, its utilization has to be declared to ensure transparency for consumers" Kaufmann et al. (57), "Therefore mTg can enhance the immunogenicity of gluten and should not be used in food products intended for consumption by CD patients" Dekking et al. (66). Not surprisingly, the worries and warnings on mTg nutritional industrial usage safety appear in numerous publications (26, 38, 57, 62, 66–68). More so, at least, in Switzerland and Germany, public warning were issued concerning mTg food safety, recommending labeling of the enzyme (69)[1]. The warnings mentioned the risk of mTg consumption by CD patients, "Suitable labeling of foods produced using mTg would enable these patients to avoid the uncertainties that the scientific community has yet to clarify"[1].

[1]http://www.bfr.bund.de/cm/343/transglutaminase-in-fleischerzeugnissen.pdf

CONCLUSIONS

Microbial transglutaminase is a food additive, heavily used in a plethora of processed food industries. It is unlabeled and hidden from the public knowledge. Being functionally similar to the tTg, it can post-translate and modify gliadin peptides by cross-linking them, thus, inducing loss of tolerance. **Figure 1** describe schematically the chain of events starting from the mTg sources ending in CD development.

There are published warnings, alarming the public on the potential danger of using or consuming this enzyme. Recent publications found mTg to be immunogenic in CD patients and its pathogenicity is continuously unraveled. The logical theoretical basis for the mTg to be a new environmental factor in CD induction exist, however, causality should further be explored.

AUTHOR CONTRIBUTIONS

MT designed, overviewed, searched, and analyzed the literature and edited the manuscript. LA designed and wrote the manuscript.

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REVIEW
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Extra-Intestinal Manifestations of Coeliac Disease in Children: Clinical Features and Mechanisms

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Celiac disease (CD) is a systemic autoimmune disease due to a dysregulated mucosal immune response to gluten and related prolamines in genetically predisposed individuals. It is a common disorder affecting ∼1% of the general population, its incidence is steadily increasing. Changes in the clinical presentation have become evident since the 80s with the recognition of extra-intestinal symptoms like short stature, iron deficiency anemia, altered bone metabolism, elevation of liver enzymes, neurological problems. Recent studies have shown that the overall prevalence of extra-intestinal manifestations is similar between pediatric and adult population; however, the prevalence of specific manifestations and rate of improvement differ in the two age groups. For instance, clinical response in children occurs much faster than in adults. Moreover, an early diagnosis is decisive for a better prognosis. The pathogenesis of extra-intestinal manifestations has not been fully elucidated yet. Two main mechanisms have been advanced: the first related to the malabsorption consequent to mucosal damage, the latter associated with a sustained autoimmune response. Importantly, since extra-intestinal manifestations dominate the clinical presentation of over half of patients, a careful case-finding strategy, together with a more liberal use of serological tools, is crucial to improve the detection rate of CD.

Keywords: extraintestinal, celiac disease, children, gluten free diet, manifestation, clinical presentation, prognosis

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INTRODUCTION

Celiac disease (CD) is a systemic autoimmune disease due to a dysregulated mucosal immune response to gluten and related prolamins, characterized by a remodeling of the small intestinal mucosa leading to villous atrophy (1), that recedes upon a gluten free diet (GFD). It occurs in genetically susceptible individuals carrying the HLA-DQ2 and/or -DQ8 and affects ∼1% of the general population in Europe, North America, North Africa, India and Middle East (2). There is evidence that its incidence is steadily increasing (3), not only because of improved awareness and more extensive use of specific diagnostic tools. Genetic factors cannot explain such a rapid increment, hence it seems to be mainly attributable to environmental factors (4).

Concomitantly with the increase in CD incidence, changes in its clinical presentation have been described since the 80s. In contrast to the "typical" presentation of CD with gastrointestinal symptoms, a higher number of asymptomatic cases has been detected by targeted screening of at-risk groups (5), as well as an increase in the number of "atypical" presentations, including extra-intestinal symptoms such as iron deficiency anemia, altered bone metabolism, short stature, and elevation of liver enzymes. A recent American study showed that non-intestinal symptoms were the most commonly represented in 43% of pediatric CD patients (6). Importantly, the use of terms such as "typical" and "atypical" to describe the clinical presentation of CD, which reflects the historical background, is currently discouraged in favor of the use of "classical" when the gastrointestinal symptoms (such as weight loss, diarrhea, distended abdomen) are prominent and "not classical" indicating cases with a predominance of extra-intestinal symptoms (7).

The overall clinical picture of CD at diagnosis became less severe (8) and the average age at diagnosis increased from below 3 years to the scholar age (9). However, most of these changes seem to have recently reached a plateau, at least as reported in Finland (9).

Recent studies showed that the prevalence of extra-intestinal manifestations is similar between the pediatric and adult population: 60 and 62%, respectively (10). However, clinical manifestations and rate of improvement differ in the two age groups. Short stature was found to be the most common feature in children, while iron deficiency anemia dominates the clinical picture in adults, furthermore, clinical symptoms in children seem to recede much faster than in adults (10, 11).

Here we reviewed the current knowledge about the extra-intestinal manifestations of CD, operating a clear distinction between extra-intestinal symptoms and CD-associated conditions. Emphasis has been given to the mechanisms underlying these pleomorphic manifestations, even though in part still unclear. Finally, we analyzed the impact of the GFD on these extra-intestinal manifestations.

ASSOCIATED DISEASES VS. EXTRA-INTESTINAL MANIFESTATIONS

There is often some confusion between the extra-intestinal symptoms and diseases associated to CD. Indeed, some clinical presentations are sometimes described as CD-associated conditions and other times as extra-intestinal manifestations, one example being dermatitis herpetiformis. The main difference is that extra-intestinal symptoms improve on a GFD, particularly if the diet is started early (12). Contrariwise, CD-associated conditions are not correlated with gluten ingestion, despite being more frequent in the CD population. In line with what observed in many autoimmune disorders, there is an association of CD with other autoimmune conditions. The recent literature reports a risk of having another autoimmune disease, in the celiac population is from 3 to 10 times more frequent than in the general population (13, 14). The most frequent associated disease is type 1 diabetes (15), that shares with CD a combination of genetic factors and common pathogenetic mechanisms. The association with other autoimmune disorders is also attributed to shared genetic risk factors, particularly the HLA genes. In addition, a possible role of gluten in triggering autoimmunity has been suggested, based on its pro-inflammatory properties and the increased risk of developing other autoimmune disorders reported in relation to the duration of gluten containing diet (16). On the contrary, the pathogenic link of CD with chromosomal abnormalities such as Down's syndrome or Turner's disease is unclear and so is for other associated conditions such as IgA deficiency.

CLINICAL MANIFESTATIONS

Short Stature

After serological screening was introduced, in the form of anti-gliadin antibodies testing, the first extra-intestinal manifestation to be identified was short stature (17). Affecting ∼10–40% of pediatric patients at the time of diagnosis (18), it still remains the most common extra-intestinal presentation of CD in children (19), sometimes being its only clinical sign (20). Up to 8% of the patients investigated for short stature will eventually receive a diagnosis of CD, that overall represents between 19 and 59% of all non-endocrinological causes of short stature (21–24). Poor growth has been more often described in children with a younger age at diagnosis and a more severe disease onset (25).

The pathogenic mechanisms underlying short stature in CD have not been fully clarified yet. Malnutrition due to malabsorption has traditionally been thought to play a major role, but more recently a multifactorial pathogenesis has been advanced. Particularly, dysfunction of the growth hormone (GH)- Insulin-like growth factor (IGF1) axis and in particular a role for ghrelin has been proposed (26). Reduced blood values of GH, IGF1, IGF-binding protein 1 and 3 (IGFBP 1, IGFBP3) and elevated levels of IGFBP2 have been reported (27). Interestingly, the exogenous administration of GH to untreated CD patients did not induce an increase in IGF-1 levels suggesting a dysfunction of growth axis associated to active CD (23). Dysregulation of the GH axis might be sustained by the elevation of pro-inflammatory cytokines such as IL-6, TNF α, interferon γ, IL-1 (28). An "autoimmune hypothesis" for short stature in CD has also been proposed (29), but evidences have been found only in few patients for whom high titers of anti-pituitary antibodies (APA) have been associated to low levels of IGF-1 (28). Hence APA titers might help identifying CD subjects with suspected GH deficiency (GHD).

The early introduction of GFD leads to a rapid growth catch-up, particularly in the first 6 months, with weight catch-up being much faster than height. Catch-up growth is a remarkable phenomenon characterized by an increase in height up to four times the average rate for the corresponding chronological age. Target height is usually reached within 3 years after diagnosis. However, sometimes CD patients do not reach their target height, possibly because a rapid catch-up growth can associate with accelerated bone maturation (27, 30, 31). When no catch-up growth can be observed despite a strict GFD, an endocrinological evaluation is mandatory to exclude GHD, condition that has been

observed in ∼0.23% of CD patients (32). Other comorbidities such as inflammatory bowel diseases (IBD), food aversion, Turner syndrome, have been reported in children with persistent short stature despite strict adherence to the GFD and should be ruled out in those cases. An early diagnosis and proper dietary regimen continuation minimize the risk of a compromised final height. Accordingly, Comba et al. (33) observed that patients who received the diagnosis of CD after the age of 6 had a significantly lower z-score for BMI, height and weight, as compared to children diagnosed at a younger age, indicating that when CD diagnosis is posed after puberty, the chances for growth catch-up are lower.

Delayed Puberty

In the pediatric untreated CD population delayed puberty is a common finding, due to hypogonadism in girls and to androgen resistance in boys (34, 35). The prevalence of delayed puberty in CD is about 11–20% (36). The pathogenesis is unclear, however a combination of nutritional deficiencies and autoimmune antibodies against hormones, their receptors and/or endocrine organs, seem to play a role (34, 35, 37). The prognosis is favorable with puberty development occurring within 6–8 months from the introduction of GFD. If the delay in puberty persists, the patient should be referred to the endocrinologist for further evaluation.

Anemia

Anemia is the most common extra-intestinal manifestation in the CD adult population, but roughly present in 15% of the CD pediatric population (10, 11, 19, 38), probably because in adults the diagnosis is delayed. The anemia is correlated with the severity of CD (39). In a recent meta-analysis, Mahadev et al. (40) observed that iron deficiency anemia is frequent in CD irrespectively of patient demographics (age and gender). Iron absorption occurs mainly in the duodenal mucosa, hence the small intestinal damage typical of active CD may lead to its malabsorption. However, the observation that anemia could be found also in children with potential celiac disease (41), seems to suggest a multifactorial pathogenesis. Anemia is most frequently due to iron deficiency, nevertheless vitamin B12 and folate deficiencies may also be responsible. Up to 84% of CD children presenting with mild anemia that strictly follow the GFD and receive iron supplementation show a complete recovery of their iron storages by 12–24 months, when their hemoglobin levels are not very low (10, 39). Oral iron formulations are often preferred over IV formulations. Recently, new oral iron formulations, sucrosomial iron, has been proposed for patients who are intolerant to iron sulfate.

Liver Abnormalities

Hypertransaminasemia has been reported as the most frequent hepatic manifestation in CD patients. Recent studies report its prevalence at about 9–14% (42). Most times the liver damage is not severe and reversible, but in rare cases it can lead to liver failure (43). The grade of hypertransaminasemia is correlated to the duodenal mucosal damage, malabsorption, and serum levels of anti-endomysial and anti-tissue transglutaminase2 (TG2) antibodies. It has been hypothesized that the altered gut permeability can determine an increased exposure to hepatotoxins in the portal circulation leading to inflammation and liver damage (19, 44, 45). Nevertheless, in line with the other extra-intestinal manifestations, also in this case autoimmune factors may play a role, as indicated by the presence of anti-TG2 antibodies deposits in the liver (46).

The response of hypertransaminasemia to a strict GFD is excellent, with a 75–95% rate of complete normalization of liver enzymes in 12–24 months (47). An early diagnosis of CD may prevent future hepatic problems and a strict compliance of GFD can make unnecessary a routine control of liver function (42). A link between thyroid and liver disease has been reported, also based on the observation that usually CD patients with elevated ALT had also a higher TSH. The liver, in fact, performs a central role in the transport, metabolism, and deiodination of thyroid hormones (42).

As far as hepatological conditions associated with CD, primary biliary cholangitis, primary sclerosing cholangitis and autoimmune hepatitis have been found overrepresented in CD patients, but in contrast to the so called "coeliac hepatitis" (elevation of liver enzymes) their course is not modified by GFD.

Bone Disease

Bone manifestations in CD patients are mainly osteopenia, defined as low bone mineral density (BMD), and osteoporosis, defined as low bone density leading to brittleness of the bones. Approximately 75% of pediatric patients have osteopenia and 10–30% osteoporosis (48). The damage of the duodenal mucosa, where both vitamin D and almost 90% of the calcium are absorbed, leads to decreased blood levels of calcium and vitamin D and consequent increased secretion of parathyroid hormone. The hyperparathyroidism is common in celiac children (12–54%) and it determines an increased bone turnover (49). Moreover, the inflammatory milieu including IL-1, TNFα, and IL-6, together with the activation of the RANK-L/RANK/osteoprotegerin pathway stimulates the bone metabolism (50, 51). The trabecular bone is usually the most involved, since it is the most metabolically active. Evaluation of the BMD is important in short-statured CD children, also in relation with height, gender, and age in order not to misinterpret BMD values. However, there is no demonstration of increased risk of fractures during childhood and youth in CD patients.

It is possible to have osteopenia also in the very early phases of the disease. A lower BMD has been observed in screening-detected CD children compared to controls; in the former, lower levels of 1-25-OH-Vitamin D and raised levels of PTH have also been measured. These values returned to the normal range after GFD was started, and that occurred already in the first year of GFD, emphasizing the importance of an early diagnosis (52, 53). Osteopenia may be further worsened by inadequate calcium intake, hence the need to supplement the GFD with Calcium-fortified foods and vitamin D metabolites (54, 55).

Joint and Musculoskeletal Disorders

The most common joint and musculoskeletal disorders in CD include myopathy, arthralgia and non-erosive arthritis that can be silent in the early stages of disease (56). The most common

finding in CD pediatric population is a subclinical synovitis, while arthralgia becomes evident with age (>12 years). The incidence of these manifestations is ∼5–10% and the most commonly involved joint is the knee, followed by the hip and the ankle (19). Ultrasonography is important for the diagnosis, particularly in those patients whose symptoms are mild. The pathogenesis of the rheumatological manifestations remains obscure (56) and similarly their response to the GFD. Iqbal and colleagues reported an improvement only in 30% of patients (57) upon gluten withdrawal, suggesting that GFD may lead to improvement of symptoms at least in a subset of patients.

It is important to remember that CD can be associated with autoimmune diseases involving joints and muscles such as Sjogren's syndrome, juvenile idiopathic arthritis, rheumatoid arthritis and systemic lupus erythematosus (LES). A two-fold risk of LES has been recently reported in the pediatric celiac population (58).

Neurological Manifestations

Several neurological manifestations are significantly associated with CD in the pediatric population. The most common being headache, that is present in up to one-fifth of the cases. Rarer conditions in the pediatric population are ataxia and neuropathy, ranging from 0.1 to 7.4%. A prevalence of 0.7–2%, not significantly different from the general population, has been described in some studies (59, 60), however other authors reported a 1.4 fold increase of epilepsy in CD children (61, 62). Thus, the link between epilepsy and CD remains still uncertain. The most common seizures patterns are the complex partial, followed by tonic-clonic seizures. A particular type of epilepsy characterized by the presence of occipital calcifications has been specifically reported in association to CD (63, 64)—**Figure 1**.

Cerebellar ataxia is more common in adults, with a median age of onset around 20 years. Chronic, symmetric distal neuropathy is the most common form of neuropathy described in CD patients, but its precise prevalence is unclear. A possible pathogenetic mechanism behind the neuropathy involves the anti-ganglioside antibodies, but nutritional deficiencies may also have a role. Particularly, some neurological manifestations are correlated with the deficit of vitamins such as E, B12 and D, or micronutrients like magnesium. Nevertheless, these manifestations may be present even in children with no enteropathy, excluding a role for malabsorption and suggesting that other mechanism might be responsible, for example a cross reaction between anti-gliadin antibodies and synapsin has been postulated. The pathogenesis of gluten ataxia can be related to the presence of anti-TG6 antibodies that might be directed against the cerebellar cells. However, their pathogenic role remains unclear since these autoantibodies can also be present in celiac children who are not affected by neurological disorders. The GFD leads to the complete recovery of headache in 76% of celiac children (65, 66) and it can be also responsible of the low prevalence of gluten ataxia and distal neuropathy when started early.

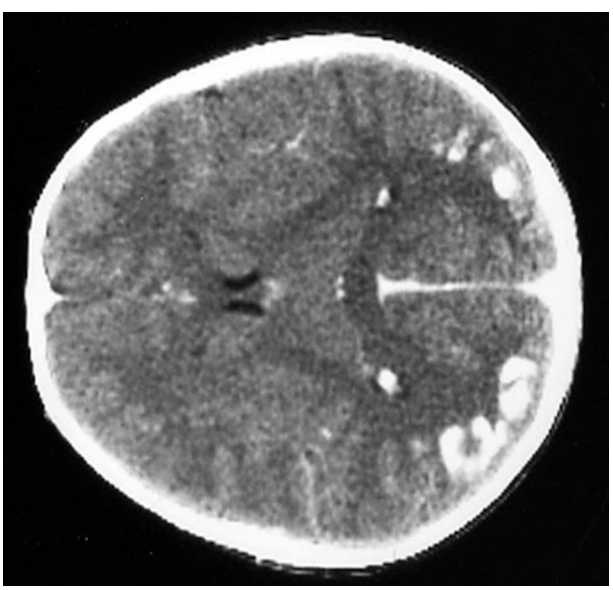

FIGURE 1 | Bilateral occipital calcifications in celiac disease.

Psychiatric Disorders

An association between CD and psychiatric disorders, including attention deficit and hyperactive disorder (ADHD), autism spectrum disorders (ASD), mood disorders, anxiety, eating disorders and depression, has been reported (67). In a large cohort of CD children, an increased risk of psychiatric disorders development (1.4-fold increase) has been observed (68). There is evidence supporting an association of CD with depression and, although to a less extent, with eating disorders (67). For panic disorder, autism and ADHD there are few reports indicating an association but further studies are necessary. Finally, the association between CD and schizophrenia or other anxiety disorders is still debated. As far as pathogenic mechanisms involved, a direct effect of CD, perhaps based on inflammation and immunological dysregulation has been proposed (69), but another possible concomitant cause could be the psychosocial discomfort associated with a chronic condition for CD children.

Enamel Defects

The exact prevalence of enamel defects in CD is not known. However, in a recent meta-analysis it was observed that CD patients had significantly higher prevalence of enamel defects compared to controls (70). Some reports indicate that it involves up to 40–50% of CD patients at diagnosis (71), but in more recent reports the percentage results to be below 15% (72) probably because clinical presentations have become less severe over time. The enamel defects are characterized by pitting and sometimes by complete loss of enamel; they include discoloration and structural changes. Aine described enamel defects as detectable in all quadrants of the dentition, involving deciduous teeth (incisors and molars are the more frequently involved teeth) and most importantly symmetrical, the latter feature being more specific for CD (73). He proposed a grading, detailed in **Table 1**. Usually defects in dental enamel occur when CD affects children during dental development (before 7 years of age). When the defect affects permanent teeth, there is no improvement upon GFD.

The defect of amelogenesis, malnutrition, in particular hypocalcemia, and immunological disturbances have been proposed as causes of enamel defects in CD. However, some reports deny a correlation between the degree of enamel defects and the severity of small bowel mucosal damage (75). Patients with HLA-DR3 genotype have been reported to have a higher risk of enamel lesions, pointing to a genetic contribution (76) (**Figure 2**).

Aphthous Stomatitis

Other oral disorders have been related to CD including delayed teeth eruption, lichen planus, cheilosis, atrophic glossitis, glossodinia. Aphthous stomatitis is an inflammatory ulcerative condition characterized by multiple recurrent small, round or ovoid ulcers with circumscribed margins appearing in the oral cavity. It usually manifests in the non-keratinized oral mucosa and can cause considerable pain. Up to 46% of CD patients have been reported to be affected by aphthous stomatitis (77). The mechanisms underlying this manifestation remain still obscure. It is unclear if there is any relation with malabsorption. Disturbances of the oral ecosystem (saliva, leukocytes, microbioma) have been hypothesized. Usually patients remit completely on GFD (10).

Alopecia

Alopecia has been observed in ∼1% of recently diagnosed CD children. Depending on the extension of the lesion it is possible to distinguish alopecia areata, totalis, and universalis. Autoimmune mechanisms are thought to be involved in its pathogenesis, nevertheless GFD can lead to the total regrowth of hair by 12–24 months in half of the cases (78).

TABLE 1 | Classification of systemic and chronologic enamel defects [modified from Aine (74)].

Grade 0	No defect
Grade 1	Enamel discoloration with yellow, cream or brown opacities and loss of normal enamel glaze.
Grade 2	Structural defect with some horizontal grooves. Change of color can be find.
Grade 3	Important structural defects with deep horizontal grooves. Discoloration may be present.
Grade 4	Destruction of tooth shape and structure. The material of enamel is fragile.

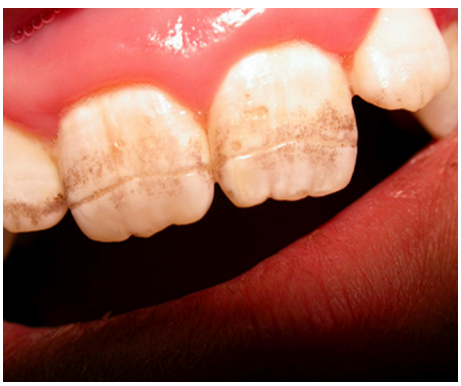

FIGURE 2 | Model of enamel defects in celiac pediatric patient; these lesions have a symmetrical distribution.

Dermatitis Herpetiformis

Dermatitis herpetiformis (DH) is considered an extraintestinal manifestation of CD. In children it is relatively rare (in Finland only 4% of all DH cases are children) (79), but in some series from other countries it seems to be more frequent in the pediatric age (80). It is particularly interesting to note that, in contrast to CD, the annual incidence rate is decreasing. It has been hypothesized that subclinical CD may predispose to DH, but what renders some individuals more prone to the development of skin lesions is unknown. The disease starts in the gut and evolves with the deposition in the papillary dermis of immune complexes of TG3 and high avidity anti-TG3 IgA antibodies (79).

From a clinical standpoint it presents with itchy papules and small blisters, often crusted because of the intense itch and consequent scratching, located on the extensor surfaces of elbows, knees and on the buttocks. Upper back, abdomen, scalp, and face may also be affected. Gastrointestinal symptoms in patients with DH are rare, but up to 72% of patients have a silent enteropathy (81). The diagnosis is made on a biopsy of unaffected skin showing by direct immunofluorescence pathognomonic granular IgA deposits at the dermo-epidermal junction. The rash recedes upon a strict GFD, with almost 100% resolution rate in children. Some patients may need additional medical therapy with dapsone, but with time the lesions are well-controlled by a GFD alone. In children the long-term prognosis on exclusion diet is excellent.

MECHANISMS UNDERLYING EXTRA-INTESTINAL MANIFESTATIONS

The pathogenesis of extra-intestinal manifestations in many respects is still unclear. There are two main mechanisms probably involved, one related to the mucosal damage and the consequent malabsorption, the second sustained by the autoimmune response. In line with the first, some extra-intestinal manifestations are clearly correlated with the severity of intestinal damage. Indeed, patients with extra-intestinal manifestations at diagnosis have a more severe grade of intestinal mucosal atrophy as compared to patients presenting only with gastrointestinal symptoms (11). Anemia is associated with malabsorption of iron, vitamin B12, and folate (82). Stunted growth is likely caused by nutrients malabsorption (25, 83). Finally, osteopenia may be due to malabsorption of calcium and vitamin D, leading to secondary hyperparathyroidism and subsequently a high bone turnover (84).

Extra-intestinal manifestations may also be the consequence of autoimmune phenomena, although in many cases this hypothesis needs to be supported by more evidences. Tissue-transglutaminase 2 (TG2) is the main, but not the only autoantigen involved in CD. Whether anti-TG2 autoantibodies play a role in CD pathogenesis has not been definitely proven.

They bind to several epitopes including the enzymatic core and can then interfere with bioactivity of TG2 (85). The presence of IgA deposits co-localizing with TG2 in liver, lymphnodes, muscle, thyroid, bone and brain (86) indicate that the autoantibodies, probably originated in the gut, can access to TG2 throughout the body and cause pathogenic effects. Consistently with this hypothesis, data in mice showed that the injection of anti-TG2 antibodies in the lateral ventricle of the brain caused deficits in motor coordination (87). Other members of transglutaminase family possibly involved in the pathogenesis of extra-intestinal manifestations in CD are TG6 and TG3. The latter is mainly expressed in the cornified layer of epidermis and for this reason also defined epidermal TG. Antibodies anti-TG3 have been found in almost 95% of DH patients representing a useful diagnostic marker for DH in both pediatric and adult patients (88). They are present also in areas located far away from the skin lesions, suggesting that other factors, besides the mere presence of the antibodies, are necessary to provoke the lesions. The TG6 is mainly expressed in neurons, playing an important role in neurogenesis (89). An association between neurological manifestation in CD and the presence of anti-TG6 has been suggested. In fact, anti-TG6 is elevated in the serum of patients with gluten neuropathy (90). Furthermore, TG6 is the target autoantigen in gluten ataxia (91). However, both specificity of these autoantibodies and gluten-dependence of their production have not been definitely proven (92).

As said, tissue transglutaminase is not the only autoantigen in CD. Antibodies to gangliosides have been reported in immune mediated peripheral neuropathies (93, 94); in particular, they have been described in CD patients in conjunction with neurological symptoms (95), their titers responding to the exclusion of gluten from the diet (96). Neutralizing autoantibodies against osteoprotegerin have also been detected in CD patients (97), but their role in development of osteoporosis is still uncertain (98). Finally, autoantibodies to cardiolipin, enolase alfa, ATP synthase beta chain, and also IgA to collagen type I, III, V, VI have been reported in CD (99), but at present there is no association/correlation between these and extra-intestinal manifestations of CD.

We reported in **Table 2** the possible pathogenetic mechanisms for each EIM in CD.

TABLE 2 | Possible pathogenetic mechanisms for each extra-intestinal manifestations in celiac disease.

Manifestation	Probable cause(s)
CUTANEOUS	
Edema	Hypoproteinemia
Dermatitis herpetiformis	Epidermal (type 3) tTG autoimmunity
ENDOCRINOLOGIC	
Amenorrhea, delayed puberty	Malnutrition, hypothalamic-pituitary dysfunction, immune dysfunction
Secondary hyperparathyroidism	Calcium and/or vitamin D malabsorption with hypocalcemia
HEMATOLOGIC	
Anemia	Iron, folate, vitamin B12, or pyridoxine deficiency
Hemorrhage	Vitamin K deficiency
HEPATIC	
Elevated liver biochemical test levels	Celiac hepatitis
Autoimmune hepatitis	Autoimmunity
MUSCULAR	
Atrophy	Malnutrition due to malabsorption
Tetany	Calcium, vitamin D, and/or magnesium malabsorption
Weakness	Generalized muscle atrophy, hypokalemia
NEUROLOGIC	
Peripheral neuropathy	Deficiencies of vitamin B12 and thiamine; immune-based neurologic dysfunction
Ataxia	Cerebellar and posterior column damage
Demyelinating central nervous system lesions	Immune-based neurologic dysfunction
Seizures	Unknown
SKELETAL	
Osteopenia, osteomalacia, and osteoporosis	Malabsorption of calcium and vitamin D, secondary hyperparathyroidism, chronic inflammation
Pathologic fractures	Osteopenia and osteoporosis
ORAL DISEASES	
Enamel hypoplasia	Vitamin D, calcium malabsorption
Aphthous stomatitis	Unknown

TREATMENT

Lifelong gluten-free diet (GFD) is the unique, effective therapy for CD (100). None of the pharmacological alternatives nowadays available or under investigation seems to be capable to replace the GFD (101).

In children GFD can lead to a complete recovery and a faster remission of extra-intestinal manifestations as compared to adults (10). The prognosis in children is very good, if adequately treated with GFD. However, timing of diagnosis is crucial. It is important to start the GFD as soon as possible. That means that an early diagnosis is decisive for a good prognosis. That is particularly true for bone diseases or for short stature. Patients diagnosed early during childhood did not have an increased risk of later development of osteoporotic fractures (102) and showed a very satisfactory catch up growth with a good final height (22). Nevertheless, sometimes the diet alone is not sufficient. This is the case of severe anemia, when it is advised to complement the dietary regimen with iron supplementation, or of severe DH, when adding a medical therapy with dapsone could be necessary in some cases (103), or even of severe osteopenia, when supplement the diet with calcium and vitamin D is important (54, 55). Problems arise when the GFD is not properly followed. This occurs mostly in adolescents (104), the pediatric population affected also by the highest prevalence of extra-intestinal manifestations and complications. When extra-intestinal manifestations show no improvement despite the GFD, patients' compliance to the dietary regimen should be questioned. In recent years a non-invasive, specific and novel approach to assess compliance with GFD has been reported, based on the detection of gluten immunogenic peptides (GIP) in the stools or in the urine by ELISA (105).

In case of no clinical improvement despite a strict GFD, additional diagnosis or pathogenic mechanism should be investigated. For instance, CD children presenting with short stature, but failing to show a satisfactory catch-up growth despite a verified compliance to the GFD, should be investigated for other co-existing conditions (e.g., growth hormone deficiency) (32) and consult the endocrinologist.

CONCLUSIONS

With extra-intestinal manifestations dominating the clinical presentation of over half of the patients, also in children CD may be considered a systemic disease. These proteiform clinical features may complicate the diagnosis. With still too many cases being undetected, education becomes very important. Given the availability of very efficient non-invasive diagnostic tools, such as measurement of serum anti-TG2 antibodies, it is necessary to increase the awareness among general pediatricians, but also other specialists (hematologists, neurologists, rheumatologists, endocrinologists). A more careful case finding strategy, together with a more liberal use of CD-specific serological tests, will improve the detection rate. Furthermore, an early diagnosis is particularly important for a faster remission of symptoms, better prognosis as well as to prevent long-term complications, especially those that are no more correctable after a certain age (e.g., osteopenia, short stature). Finally, extra-intestinal manifestations may help understanding the pathogenic mechanisms of CD, in particular the role played by autoimmunity in the different clinical presentations.

AUTHOR CONTRIBUTIONS

SN: first draft of the manuscript, manuscript revision, final approval of the version to be published, and agreement to be accountable for all aspects of the work. RT: corresponding author, primary responsibility for communication with the journal during the manuscript submission, drafting the work, manuscript revision, final approval of the version to be published, agreement to be accountable for all aspects of the work. RA and VD: critical revision of the article, final approval of the version to be published, agreement to be accountable for all aspects of the work.

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Dual Role of *PTPN22* but Not *NLRP3* Inflammasome Polymorphisms in Type 1 Diabetes and Celiac Disease in Children

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Genetic polymorphisms in genes coding for inflammasome components *nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3)* and *caspase recruitment domain-containing protein 8 (CARD8)* have been associated with autoinflammatory and autoimmune diseases. On the other hand several studies suggested that NLRP3 inflammasome contributes to maintenance of gastrointestinal immune homeostasis and that activation of NLRP3 is regulated by protein tyrosine phosphatase non-receptor 22 (PTPN22). *PTPN22* polymorphism was implicated in the risk for various autoimmune diseases including type 1 diabetes (T1D) but not for celiac disease (CD). The aim of our study was to evaluate the role of inflammasome related polymorphisms in subjects with either T1D or CD as well as in subjects affected by both diseases. We examined *PTPN22* rs2476601 (p.Arg620Trp), *NLRP3* rs35829419 (p.Gln705Lys), and *CARD8* rs2043211 (p.Cys10Ter) in 66 subjects with coexisting T1D and CD, 65 subjects with T1D who did not develop CD, 67 subjects diagnosed only with CD and 127 healthy unrelated Slovenian individuals. All results were adjusted for clinical characteristic and human leukocyte antigen (HLA) risk. *PTPN22* rs2476601 allele was significantly more frequent among subjects with T1D ($P_{adj} = 0.001$) and less frequent in subjects with CD ($P_{adj} = 0.039$) when compared to controls. In patients with coexisting T1D and CD this variant was significantly less frequent compared to T1D group ($P_{adj} = 0.010$). Protective effect on CD development in individuals with T1D was observed only within the low risk HLA group. On the other hand, we found no association of *NLRP3* rs35829419 and *CARD8* rs2043211 with the development of T1D, CD or both diseases together. In conclusion *PTPN22* rs2476601polymorphism was significantly

associated with the risk of developing T1D in Slovenian population, while no associations of proinflammatory *NLRP3* and *CARD8* polymorphisms with T1D and CD were observed. Interestingly, the same *PTPN22* variant protected from CD. We hypothesize that this effect may be mediated through the NLRP3 inflammasome activation.

Keywords: inflammasome, *NLRP3*, *CARD8*, *PTPN22*, type 1 diabetes, celiac disease

INTRODUCTION

Type 1 diabetes (T1D) is characterized by chronic autoimmune mediated destruction of pancreatic β-cells, leading to symptomatic partial, or in most cases, absolute insulin deficiency requiring lifelong administration of exogenous insulin (1). Celiac disease (CD) is a chronic immune-mediated disorder characterized by the presence of variable combination of clinical manifestations ranging from enteropathy to a systemic affliction of different organ systems and appearance of autoimmune antibodies elicited by ingestion of gluten and related prolamines (2). Both multifactorial diseases have a strong autoimmune and genetic component and often segregate together (3). Because of common association of T1D with CD, screening for CD is recommended in all children with T1D (1, 2). In individuals with T1D positive serum anti-tissue transglutaminase (tTG) antibodies may spontaneously normalize even on a gluten-containing diet (4). Therefore, duodenal biopsy is still required in asymptomatic individuals with T1D to confirm the diagnosis of CD (2). The majority of patients develop CD after the onset of T1D and the risk is significantly higher in children with T1D diagnosed before 5 years of age (5). In addition to CD autoimmune thyroid disease (ATD) is frequently seen in individuals with T1D (6). Having T1D and ATD has been reported to be a risk factor for the development of CD (5). On the other hand children affected by CD were at increased risk of subsequent hypothyroidism (7).

Shared susceptibility alleles in the Human leukocyte antigen (HLA) region encoding HLA-DQ2 and DQ8 molecules which account for much of the etiology of T1D and CD contribute to the coexistence of both diseases. The risk is further modified by the HLA genotype, which is the combination of HLA alleles inherited from both parents (8, 9). In our previous study, *DR3-DQ2/DR4-DQ8* and *DR3-DQ2/DR3-DQ2* genotypes were found to be highly predisposing for developing both diseases together (10). However, disease co-occurrence is greater that could be accounted for HLA-DR-DQ genotypes. In the prospective birth cohort study persistent islet autoantibodies were associated with increasing risk of subsequent development tTG antibodies, suggesting that T1D autoimmunity is a risk factor for subsequent CD (11). Cumulative genetic risk associated with both T1D and CD increases substantially when combined with the effect of genetic polymorphisms outside the HLA region (12). Among non-HLA factors shared pathophysiological mechanisms may involve many genes of the innate immune signaling, which is the first line of defense against invading microorganisms not only by directly eliminating the pathogen but also through further shaping of the adaptive immunity (13). It is hypothesized that the environment increasingly conducive to T1D development may potentiate autoimmunity by dysregulating innate immune processes in genetically susceptible individuals (14, 15). The inflammasomes are highly conserved pattern-recognition receptors expressed by cells of the innate immune system and some tissue cells. These cells recognize microbial pathogens as well as cellular stress and damage, leading to production of pro-inflammatory cytokines interleukin (IL)-1β and IL-18 and triggering a process of inflammation-related cell death (16). The best characterized is the NLRP3 inflammasome, the protein complex including nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) protein, adapter protein apoptosis-associated speck-like protein and procaspase-1. Interactions among these proteins tightly regulate inflammasome function in order to ensure immune activity only when appropriate (17). *NLRP3* rs35829419 (p.Gln705Lys) is a gain-of-function polymorphism associated with pro-inflammatory phenotype (18, 19). Caspase recruitment domain-containing protein 8 (CARD8) negatively regulates NLRP3 inflammasome through its binding with NLRP3 protein. *CARD8* rs2043211 (p.Cys10Ter) results in non-functional protein and leads to loss of CARD-8 inhibition of caspase-1 (20). Aberrant NLRP3 inflammasome activation has been linked to various immune disorders (21) and can also contribute to the onset and progression of metabolic diseases (22, 23). It was shown that NLRP3 played an important role in the immune pathogenesis of T1D development in NOD mice (24). It seems that NRLP3 also plays a regulatory and reparative role in the maintenance of immune tolerance and intestinal epithelial barrier integrity (25).

Tyrosine phosphorylation is an important regulatory mechanism that prevents aberrant inflammasome activation. A recent study has shown that protein tyrosine phosphatase non-receptor 22 (PTPN22) interacts with and dephosphorylates NLRP3 upon proinflammatory insults, allowing robust NLRP3 activation and IL-1β secretion (26). The *PTPN22* rs2476601 (p.Arg620Trp) polymorphism has been linked to several autoimmune diseases (27). The association between *PTPN22* rs2476601 and T1D was initially identified by Bottini et al. in two independent populations (28). Interestingly, the results of comprehensive meta-analysis showed no or weak association of *PTPN22* rs2476601 with CD (29, 30) and some autoimmune diseases primarily affecting the gastrointestinal system (31).

Abbreviations: T1D, type 1 diabetes; CD, celiac disease; T1D+CD, coexisting T1D and CD; HLA, human leukocyte antigen; *NLRP3, nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3; CARD8, caspase recruitment domain-containing protein 8; PTPN22, protein tyrosine phosphatase non-receptor 22*; LR, low-risk; HR, high-risk.

Previous studies have shown conflicting and heterogeneous findings with regard to the role of *NLRP3* and *PTPN22* polymorphisms in either T1D or CD onset. However, T1D and CD frequently occur in the same individual, pointing to shared mechanisms. The aim of our study was to investigate the role of *PTPN22* rs2476601 (p.Arg620Trp), *NLRP3* rs35829419 (p.Gln705Lys), and *CARD8* rs2043211 (p.Cys10Ter) in subjects with T1D who did not develop CD, in subjects with CD who did not develop T1D and in subjects who developed both diseases. Furthermore, the role of these polymorphisms was assessed while also taking into account the presence of high or low risk HLA genotype.

MATERIALS AND METHODS

Study Design and Subjects

In total 325 individuals, who were previously HLA typed (10), were included in our study. Patients were stratified into three groups: 66 with coexisting T1D and CD (T1D+CD), 65 with T1D who did not develop CD after minimum of 10 years of follow up and 67 diagnosed only with CD. A group of 127 healthy unrelated Slovenian individuals served as a control group. In all subjects CD was diagnosed according to the relevant European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria (2, 32). In individuals with T1D serological screening for CD started at the onset of T1D and continued yearly. Study included 862 children from 0 to 17.99 years of age at diagnosis, referred to the University Children's Hospital Ljubljana, Slovenia at the onset of T1D, from 1995 to 2009. Screening for celiac disease was based on the detection of endomysial immunoglobulin A (IgA) autoantibodies (EMA) measured by indirect immunofluorescence until 2000. Afterwards tTG measured by enzyme linked immunosorbent assay were used for screening. Total serum IgA was used to exclude IgA deficiency. In IgA-deficient individuals IgG-specific anti-bodies (tTG and/or EMA IgG) were detected. Since individuals with T1D may have fluctuating levels of CD-specific antibodies, the definitive diagnosis of CD was established by an intestinal biopsy sample showing a Marsh score of 2 or higher in 69 participants. This study included 66 participants for whom DNA samples were available. Most of the subjects were diagnosed with CD after T1D diagnosis (designated as T1D first), while 22 (33.8%) had CD before T1D onset or were diagnosed with CD at the time of T1D diagnosis as the result of the screening (designated as CD first).

PTPN22, NLRP3, and *CARD8* Genotyping

For the detection of *PTPN22* rs2476601 (c.1858T>C) polymorphism we used TaqMan SNP Genotyping assay (ID:C_16021387_20; Applied Biosystems, Foster City, CA, US). *NLRP3* rs35829419 (c.2113C>A, p.Gln705Lys) and *CARD8* rs2043211 (c.304A>T; p.Phe102Ile, p.Cys10Ter) were genotyped using a fluorescence-based competitive allele-specific (KASPar) assay according to the manufacturer's instructions (KBiosciences, Herts, UK). Genotyping was repeated in 20% of samples to check for genotyping reliability.

HLA DRB1, DQB1, and *DQA1* Typing

The *DRB1, DQB1,* and *DQA1* genotyping was performed as previously described (10). We performed PCR-sequence-specific primer (PCR-SSP) typing (Olerup SSP, Stockholm, Sweden), PCR-reverse sequence-specific oligonucleotide probe (PCR-rSSO) typing assay (LabType SSO, One Lambda, Kanoga Park, CA, USA) with Luminex technology (LABSCAN TM 100, Luminex corporation, Austin, TX, USA) and PCR-sequencing based typing (PCR-SBT) (Conexio's SBT Resolver™, Conexio-Genomics, Fremantle, Western Australia).

Statistical Analysis

HLA alleles were deduced into three loci haplotypes (*DRB1-DQA1-DQB1*) as described earlier (10). DRB1*03:01-DQA1*05:01-DQB1*02:01 haplotype was abbreviated as DR3-DQ2, DRB1*07:01–DQA1*02:01-DQB1*02:02 as DR7-DQ2 and DRB1*11/12–DQA1*05:05–DQB1*03:01 as DR5-DQ7. DRB1*04:01-DQA1*03:01-DQB1*03:02, DRB1*04:02-DQA1*03:01-DQB1*03:02, DRB1*04:04-DQA1* 03:01-DQB1*03:02, DRB1*04:05-DQA1*03:01-DQB1*03:02, and DRB1*04:08-DQA1*03:01-DQB1*03:04 haplotypes were considered as DR4-DQ8. X was defined as non-DR3-DQ2 and non-DR4-DQ8 haplotype. *HLA-DRB1-DQA1-DQB1/DRB1-DQA1-DQB1* genotypes (combination of two *DRB1-DQA1-DQB1* haplotypes) were grouped into two HLA-risk categories encoded as low-risk (LR) and high-risk (HR) categorical variables by means of the known risk associated with T1D and CD in the Slovenian population. The heterozygous genotype DR3-DQ2/DR4-DQ8 and genotype DR4-DQ8/ DR4-DQ8 were defined as HR for T1D (8, 33, 34). Since the HLA influence on CD susceptibility showed a dose effect, DR3-DQ2/ DR3-DQ2 and DR3-DQ2/ DR7-DQ2, which are *DQB1*02* homozygous combinations were classified as high risk for CD (10). DR3-DQ2/X *and* DR4-DQ8/X single dose combinations, DRB1*07-DQA1*02:01-DQB1*02:02/DRB1*11/12-DQA1*05:05-DQB1*03:01 haplotype combination with DQ2 alleles carried in trans as well as non-DQ2 and non-DQ8 (X/X) haplotype combinations were considered as low risk.

For the description of continuous and categorical variables, median with interquartile range and frequencies were used, respectively. Standard chi square test was used to compare distribution of categorical clinical characteristics between different patient groups and to assess deviation from Hardy-Weinberg equilibrium. Nonparametric Mann-Whitney and Kruskal-Wallis tests were used to compare continuous clinical characteristics between different patient groups. Logistic regression was used for comparison of genotype frequencies, calculating odds ratio (OR) and 95% confidence interval (CI), as well as for the analysis of interaction between polymorphisms. Two-tailed Fisher's exact test was used for comparisons of the frequencies of HLA deduced genotypes. The level of statistical significance was set at 0.05. All statistical analyses were performed using IBM SPSS Statistics 19.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Clinical Characteristics of Subjects With T1D, CD, and T1D+CD

Clinical characteristics of subjects with T1D, CD, or both including HLA-risk categories are shown in **Table 1**. Subjects with CD were the youngest at the time of the first diagnosis, but the difference did not reach statistical significance when all three groups were compared ($P = 0.072$, **Supplementary Figure 1**). Antithyroid antibodies were observed in 18 individuals with T1D (27.7%) and in 16 individuals (24.2%) with coexisting T1D and CD with no significant difference between the two groups. Three individuals (4.5%) were diagnosed with ATD in the CD only group (**Table 1**).

HLA-DRB1-DQA1-DQB1 Deduced Genotype Frequencies in Individuals With Coexisting T1D and CD

The distribution of HLA genotypes differed significantly among the groups (**Supplementary Table 1**). Two high-risk genotypes DR3-DQ2/DR4-DQ8 ($p < 0.001$) and DR3-DQ2/DR3-DQ2 ($p < 0.001$) were associated with an increased risk for developing the combination of both diseases. On the other hand low risk genotypes had neutral effect on co-occurrence. When individuals with T1D who later developed CD were compared to those with T1D alone, genotype DR3-DQ2/DR3-DQ2 (OR = 7.10, $p = 0.002$) conferred an increased risk for CD and DR4-DQ8/X (OR = 0.19, $p = 0.001$) was negatively associated. Comparisons of individuals in whom CD diagnosis proceeded T1D with CD only group revealed that DR3-DQ2/DR4-DQ8 genotype was associated with an increased risk for developing T1D (OR = 9.96, $p = 0.002$).

TABLE 1 | Characteristics of subject with T1D, CD, and T1D+CD.

Characteristic	T1D *N* = 65	CD *N* = 67	T1D + CD *N* = 66
GENDER			
Male *N* (%)	32 (49.2)	23 (34.3)	32 (48.5)
Female *N* (%)	33 (50.8)	44 (65.7)	34 (51.5)
AGE AT T1D DIAGNOSIS			
Median (25–75%)	7.8 (3.6–11.4)		8.6 (4.0–11.8)
AGE AT CD DIAGNOSIS			
Median (25–75%)		4.8 (2.3–9.5)	10.0 (5.0–14.9)
CD DIAGNOSIS BEFORE/AT T1D DIAGNOSIS			
[a]*N* (%)			22 (33.8)
ATD (%)	18 (27.7)	3 (4.5)	16 (24.2)
HLA GENOTYPE			
LR *N* (%)	39 (60.0)	43 (64.2)	28 (42.4)
HR *N* (%)	26 (40.0)	24 (35.8)	38 (57.6)

T1D, type 1 diabetes; CD, celiac disease; T1D+CD, coexisting T1D and CD; ATD, autoimmune thyroid disease; HR, high risk HLA genotype for T1D or CD; LR, low risk HLA genotype for T1D or CD.

[a]*Data on first diagnosis is missing for 1 subject with coexisting T1D and CD.*

PTPN22, NLRP3, and *CARD8* Genotype Frequencies Distribution

Genotype frequencies were in agreement with HWE in the control group ($P = 0.413$ for *PTPN22* rs2476601, $P = 0.461$ for *NLRP3* rs35829419, and $P = 0.616$ for *CARD8* rs2043211) as well as in the entire study group ($P = 0.823$ for *PTPN22* rs2476601, $P = 0.138$ for *CARD8* rs2043211, and $P = 0.607$ for *NLRP3* rs35829419). Minor allele frequencies were 14.2% for *PTPN22* rs35829419, 38.2% for *CARD8* rs2043211, and 6.6% for *NLRP3* rs2476601 (**Table 2**).

Association Between *PTPN22*, *NLRP3*, and *CARD8* Genotype Frequencies and Risk of T1D, CD, and T1D+CD

Proportion of carriers of at least one polymorphic *PTPN22* rs2476601allele was significantly higher among subjects withT1D (OR = 2.65, 95% CI = 1.41–5.00, $P = 0.003$) and the difference remained statistically significant after adjustment for gender (OR = 3.02, 95% CI = 1.54–5.93, $P = 0.001$). On the other hand, polymorphic *PTPN22* rs2476601allele was significantly less common in subjects with CD when compared to controls (OR = 0.36, 95% CI = 0.15–0.87, $P = 0.024$), the difference remained significant after adjustment for gender (OR = 0.39, 95% CI = 0.16–0.96, $P = 0.039$). There were no significant differences in genotype frequencies for *NLRP3* rs35829419 and *CARD8* rs2043211 between cases and controls, not even after adjustment for clinical parameters (**Table 3**).

Comparison of *PTPN22, NLRP3,* and *CARD8* Genotype Frequencies of Subjects With T1D, CD, and T1D+CD

Proportion of carriers of at least one polymorphic *PTPN22* rs2476601allele in subjects with coexisting T1D and CD was significantly lower compared to T1D (OR = 0.41, 95% CI = 0.19–0.85, $P = 0.016$) and the difference remained significant after adjustment for age at diagnosis, gender and HLA genotype (OR = 0.36, 95% CI = 0.17–0.78, $P = 0.010$). Proportion of carriers of at least one polymorphic *PTPN22* rs2476601 allele in subjects with coexisting T1D and CD was significantly higher compared to CD (OR = 2.97, 95% CI =1.14–7.75, $P = 0.026$), however the difference was no longer significant after adjustment for age at diagnosis, gender and HLA genotype (OR = 2.42, 95% CI = 0.82–7.12, $P = 0.109$). *NLRP3* rs35829419 and *CARD8* rs2043211 genotype frequencies did not differ significantly among subjects with T1D, CD, or coexisting diseases (**Table 4**). No significant differences in genotype frequencies were observed when subjects with coexisting T1D and CD were stratified according to the order of the disease onset (T1D first and CD first groups) and compared to patients with T1D only and CD only as well as when compared between themselves (**Table 4**).

Multiplicative Interaction

When comparing subjects that were first diagnosed with T1D and then developed CD (T1D first) to patients with only T1D, we observed a multiplicative interaction between *PTPN22* and

TABLE 2 | Genotype frequencies of *CARD8*, *NLRP3,* and *PTPN22* polymorphisms.

Polymorphism	Genotype	All individuals *N* (%)	Controls *N* (%)	T1D *N* (%)	CD *N* (%)	T1D + CD *N* (%)	T1D first[a] *N* (%)	CD first[a] *N* (%)
***CARD8* rs2043211**	AA	118 (36.3)	51 (40.2)	26 (40.0)	20 (29.9)	21 (31.8)	15 (34.9)	6 (27.3)
	AT	166 (51.1)	61 (48.0)	31 (47.7)	36 (53.7)	38 (57.6)	25 (58.1)	13 (59.1)
	TT	41 (12.6)	15 (11.8)	8 (12.3)	11 (16.4)	7 (10.6)	3 (7.0)	3 (13.6)
***NLRP3* rs35829419[b]**	CC	283 (87.3)	111 (88.1)	59 (90.8)	58 (86.6)	55 (83.3)	36 (83.7)	18 (81.8)
	CA	39 (12.0)	14 (11.1)	5 (7.7)	9 (13.4)	11 (16.7)	7 (16.3)	4 (18.2)
	AA	2 (0.6)	1 (0.8)	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
***PTPN22* rs2476601**	CC	240 (73.8)	96 (75.6)	35 (53.8)	60 (89.6)	49 (74.2)	30 (69.8)	18 (81.8)
	CT	78 (24.0)	30 (23.6)	29 (44.6)	6 (9.0)	13 (19.7)	12 (27.9)	1 (4.5)
	TT	7 (2.2)	1 (0.8)	1 (1.5)	1 (1.5)	4 (6.1)	1 (2.3)	3 (13.6)

T1D, type 1 diabetes; CD, celiac disease; T1D+CD, coexisting T1D and CD; T1D first, patients who first developed T1D and later CD; CD first, patients who first developed CD and later T1D.

[a] *Data on first diagnosis is missing for 1 subject with coexisting T1D and CD.*

[b] *Data is missing for 1 control.*

TABLE 3 | Association between *CARD8, NLRP3, and PTPN22* genotype frequencies and risk of T1D, CD and T1D+CD.

		T1D vs. controls		CD vs. controls		T1D+CD vs. controls	
		OR (95% CI)	*P*[a]	OR (95% CI)	*P*[a]	OR (95% CI)	*P*[a]
CARD8 rs2043211	AT	0.96 (0.49–1.87)	0.900	1.45 (0.75–2.82)	0.274	1.34 (0.68–2.64)	0.397
	TT	1.11 (0.40–3.08)	0.842	1.89 (0.73–4.84)	0.188	1.18 (0.41–3.43)	0.761
	AT+TT	0.99 (0.52–1.87)	0.967	1.54 (0.81–2.91)	0.187	1.31 (0.62–2.52)	0.417
NLRP3 rs35829419	CA+AA	0.76 (0.27–2.15)	0.609	1.20 (0.49–2.93)	0.692	1.53 (0.64–3.68)	0.343
PTPN22 rs2476601	CT+TT	3.02 (1.54–5.93)	0.001	0.39 (0.16–0.96)	0.039	1.25 (0.61–2.56)	0.543

T1D, type 1 diabetes; CD, celiac disease; T1D+CD, coexisting T1D and CD.

[a] *Adjusted for gender.*

HLA (OR = 7.19, 95% CI = 1.03–50.29, $P = 0.047$) (**Table 5**). Subjects with LR HLA and *PTPN22* CC genotypes were the reference category. Carriers of at least one polymorphic *PTPN22* allele were significantly less frequent among subjects withT1D first compared to T1D only within HLA LR group ($P = 0.015$), but not in the HLA HR group. Among carriers of at least one polymorphic *PTPN22* allele, T1D first were more likely HLA HR than T1D ($P = 0.014$). A similar trend was observed when comparing group of subjects with both T1D and CD (T1D first + CD first) to subjects with only T1D or CD, but it did not reach statistical significance. No multiplicative interaction was observed between *NLRP3* rs35829419 and *CARD8* rs2043211 (**Supplementary Table 2**).

DISCUSSION

The present study investigated the role of *PTPN22* and inflammasome polymorphisms in the risk for development of T1D and CD as separate or concurrent diseases. Our results suggest a dual role of the *PTPN22* polymorphism in the development of autoimmune diseases: while rs2476601 polymorphism was associated with increased risk for T1D, it was protective against development of CD. On the other hand, *NLRP3* and *CARD8* polymorphisms were not associated with the risk of either T1D or CD.

Previous studies reported associations of *PTPN22* and *NLRP3* polymorphisms with several autoimmune diseases suggesting their general role in the etiology of autoimmunity, however these risk alleles are not shared among all autoimmune disease (31, 35). NOD mice models showed an important role of aberrant NLRP3 activation in the immune pathogenesis of T1D (24). In preclinical studies NLRP3 deficient animals have been shown to have defective epithelial barrier function and increased intestinal inflammation (36). In human studies of CD, peripheral blood mononuclear cells and monocytes responded to gliadin fraction by a robust secretion of IL-1β and IL-1α and a slightly elevated production of IL-18 (37). In contrast with these functional studies, our results did not

TABLE 4 | Comparison of *CARD8*, *NLRP3,* and *PTPN22* genotype frequencies of subjects with T1D, CD, and T1D+CD.

		T1D+CD vs. T1D		T1D+CD vs. CD		T1D first vs. T1D		CD first vs. CD		CD first vs. T1D first	
		OR (95% CI)	*P*[a]	OR (95% CI)	*P*[b]	OR (95% CI)	*P*[a]	OR (95% CI)	*P*[b]	OR (95% CI)	*P*[c]
CARD8 rs2043211	AT	1.39 (0.65–2.98)	0.402	0.89 (0.38–2.09)	0.786	1.24 (0.53–2.88)	0.622	1.32 (0.41–4.28)	0.641	1.68 (0.49–5.76)	0.406
	TT	0.97 (0.30–3.17)	0.954	0.58 (0.16–2.10)	0.411	0.60 (0.14–2.68)	0.504	0.99 (0.19–5.10)	0.994	2.45 (0.36–16.81)	0.362
	AT+TT	1.30 (0.62–2.71)	0.486	0.82 (0.36–1.87)	0.636	1.11 (0.49–2.51)	0.808	1.25 (0.40–3.88)	0.705	1.80 (0.55–5.90)	0.334
NLRP3 rs35829419	CA+AA	2.01 (0.68–5.95)	0.210	1.44 (0.50–4.09)	0.500	1.89 (0.57–6.25)	0.299	1.22 (0.31–4.79)	0.778	1.08 (0.27–4.34)	0.917
PTPN22 rs2476601	CT+TT	0.36 (0.17–0.78)	0.010	2.42 (0.82–7.12)	0.109	0.47 (0.20–1.11)	0.085	1.88 (0.48–7.41)	0.367	0.25 (0.11–1.77)	0.251

HR, high risk HLA genotype; LR, low risk HLA genotype; T1D, type 1 diabetes; CD, celiac disease; T1D+CD, coexisting T1D and CD; T1D first, patients who first developed T1D and later CD; CD first, patients who first developed CD and later T1D.

[a]*Adjusted for gender, age at T1D diagnosis, HLA HR vs. LR.*

[b]*Adjusted for gender, age at CD diagnosis, HLA HR vs. LR.*

[c]*Adjusted for gender, age at first diagnosis, HLA HR vs. LR.*

TABLE 5 | Interaction between *PTPN22* rs2476601 and HLA (comparing T1D first to T1D).

	HLA								HLA within one *PTPN22* category	
	LR				HR					
PTPN22	**T1D first (*N*)**	**T1D (*N*)**	**OR (95% CI)**	***P***	**T1D first (*N*)**	**T1D (*N*)**	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
CC	17	21	1	Ref.	13	14	1.15 (0.43–3.09)	0.786	1.15 (0.43–3.09)	0.786
CT+TT	2	18	0.14 (0.03–0.68)	0.015	11	12	1.13 (0.40–3.20)	0.815	8.25 (1.55–44.02)	0.014
PTPN within one HLA category			0.14 (0.03–0.68)	0.015			0.99 (0.32–3.00)	0.982		

T1D, type 1 diabetes; CD, celiac disease; T1D+CD, coexisting T1D and CD; T1D first, patients who first developed T1D and later CD; HR, high risk HLA genotype; LR, low risk HLA genotype.

confirm the role of functional *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms that promote NLRP3 inflammasome activation in the risk of either T1D or CD. Conflicting results were also reported in the literature, as the minor *NLRP3* rs35829419 A allele was reported to be associated with CD in Brazilian population (38), however in Italian patients this allele appeared to have a protective role against the development of CD (39).

Our finding that *PTPN22* polymorphism has a dual role in the development of autoimmune diseases is of particular interest as recent studies showed that NLRP3 activation is negatively regulated by tyrosine phosphorylation (40). The presence of autoimmunity associated PTPN22 variant in mice (PTPN22-619W) resulted in dephosphorylation and activation of NLRP3, while loss of PTPN22 resulted in decreased NLRP3 mediated IL-1β secretion. PTPN22 affected NLRP3 activation only in the lamina propria, resulting in only minor changes of IL-18 activation, which is mainly produced by intestinal epithelial cells. Furthermore, loss of PTPN22 resulted in aggravated intestinal inflammation (26). Of note *PTPN22* c.1858C>T variant leading to p.Arg620Trp substitution (rs2476601) showed association pattern with target tissue specificity being less involved in pathogenesis of autoimmune diseases triggered by environmental factors primarily acting on target tissues such as gut mucosa (31). Supporting the notion of negligible effect of *PTPN22* rs2476601 on CD development, no statistically significant association of this polymorphism with CD was found in a north European (41, 42) and Spanish population (29). Nevertheless, we observed an apparent protective effect of *PTPN22* rs2476601 polymorphism on CD development, suggesting a role of PTPN22 in facilitating inflammasome activation in intestinal lamina propria.

Previous studies have consistently associated *PTPN22* rs2476601 with the T1D onset in several populations (43). A definite effect of this polymorphism on the appearance of autoantibodies and progression to clinical T1D was observed

in the DIPP study cohort, where the carriers of the *PTPN22* rs2476601 polymorphism showed enhanced appearance of T1D associated autoantibodies when exposed to cow milk in early infancy (44). Consistent with these reports we observed significantly higher frequency of carriers of at least one rs2476601 T allele among subjects withT1D without associated CD as compared to the control group supporting a role of this polymorphism in disease susceptibility in the Slovenian population. Given a role of PTPN22 in pathogenesis of the T1D and lack of association to CD we also investigated the role of *PTPN22* in T1D with accompanying CD. However, unlike to the individuals with isolated T1D, *PTPN22* rs2476601 was not associated with increased risk for T1D with accompanying CD. These results suggest that *PTPN22* rs2476601 may not influence T1D pathophysiological mechanisms in all cases, while previous reports on the association of this variant and APSIII onset suggest common pathogenic pathways (45). The discrepancies observed among studies may in part be explained by considerable differences of *PTPN22* rs2476601 frequencies in populations of European origin (31). Minor allele frequency for *PTPN22* rs2476601 in our population was 14.2%, resembling more closely those from northern European populations, which are among the highest in Europe (46).

Since HLA remains, by far, the strongest predictor of T1D and CD risk all results in our study were adjusted for HLA risk and only odds ratios adjusted for given HLA risk category were reported. Previous studies have shown that the strength of association between *PTPN22* rs2476601 and T1D is stronger in low-risk HLA subjects, compared with that in subjects caring high-risk HLA genotypes (47). In our study, the interaction effect of HLA-risk category on the association of *PTPN22* rs2476601 was observed when individuals who had first T1D and then developed CD (T1D first group) were compared to individuals with T1D who did not develop CD. Protective effect on CD development was observed within the low risk HLA category but no significant difference was observed between groups within the HLA high risk category. More than half of patients who had coexisting T1D and CD carried the high risk HLA-DR3-DQ2/DR3-DQ2 or HLA-DR3-DQ2/DR4-DQ8 genotype. On the other hand, DR4-DQ8/X was protective for developing CD in individuals with T1D. If the results could be confirmed on a larger number of patients of different ethnic origin, assessing the presence of HLA-DQ2 and DQ8 genotypes in individuals negative for celiac disease-specific antibodies at the time of T1D diagnosis could be considered as an algorithm tool to optimize further screening frequency. Yearly screening for CD may be considered in T1D individuals with high risk HLA-DR3-DQ2/DR3-DQ2 or HLA-DR3-DQ2/DR4-DQ8 genotypes. On the other hand, individuals carrying DR4-DQ8/X genotype could be further genotyped for PTPN22 rs2476601 (c.1858T>C) polymorphism and if positive, less frequent screening for CD may be suggested.

The small sample size was a limitation of our study. The possibility of insufficient power to detect associations of genetic variants with small effect on disease susceptibility must be considered. Our results suggest opposite effects of *PTPN22* variant on T1D and CD susceptibility and provide interesting information on incidence of this variant in subjects diagnosed with both autoimmune diseases together. To better understand the complex effect of genetic factors and their regulatory role in the pathogenesis of autoimmune diseases larger studies and functional studies are warranted. Due to the low prevalence of subjects with concurrently diagnosed T1D and CD the main advantage of our study was the use of longitudinal data (48) on the entire pediatric T1D population form the prospective Slovene childhood-onset T1D register (49) from 1995 to 2009, which allowed a longer follow up, up to 10 years for T1D only group.

In conclusion this study showed that *PTPN22* rs2476601 polymorphism was significantly associated with the risk of developing T1D in Slovenian population, and of interest, the same variant protected from CD. We hypothesize that the underlying mechanism for this controversial effect could be explained considering that NLRP3 activation is involved in maintaining intestinal homeostasis. However, no association of proinflammatory *NLRP3* and *CARD8* polymorphisms with CD were observed. Interestingly, *PTPN22* rs2476601 did not influence T1D susceptibility in individuals with coexisting CD, suggesting heterogeneity in the disease-predisposing effect of *PTPN22* rs2476601 in T1D with regard to the presence of CD. Although individuals with coexisting T1D and CD represent only a small percentage of all T1D cases, acknowledging the diversity of genetic background may help to improve immune intervention strategies aimed at curing T1D.

STATEMENT OF PRIOR PRESENTATION

Part of this study was presented as a poster with a title of "Polymorphism c1858t in the protein tyrosine phosphatase non-receptor type 22 in co-occurrence of type 1 diabetes and celiac disease" (Abstract Code: A-910-0012-00527) at the 43rd Annual Conference of the International Society for Pediatric and Adolescent Diabetes in Innsbruck, Austria, October 18–21, 2017.

ETHICS STATEMENT

The study protocol was approved by the Republic of Slovenia National Medical Ethics Committee (No. 98/09/12) and written informed consent was obtained from all participating individuals and/or their parents, when appropriate, prior to the enrolment.

AUTHOR CONTRIBUTIONS

DS collected clinical data, interpreted data, and drafted the manuscript. KG carried out the *NLRP3* and *CARD* molecular genetic studies, statistical analysis, and edited the manuscript. TH carried out the *PTPN22* molecular genetic studies. AM and BV-J performed analysis of the HLA genetic studies. NB and JB were responsible for the clinical data and biological sample

collection used in this study. VD and TB contributed to the study design and conception and edited the manuscript. All the authors collaborated in revising the manuscript and accepted the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00063/full#supplementary-material

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REVIEW
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Evolving Therapy for Celiac Disease

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Gluten is known to be the main triggering factor for celiac disease (CeD), an immune-mediated disorder. CeD is therefore managed using a strict and lifelong gluten-free diet (GFD), the only effective treatment available currently. However, the GFD is restrictive. Hence, efforts are being made to explore alternative therapies. Based on their mechanisms of action on various molecular targets involved in the pathogenesis of CeD, these therapies may be classified into one of the following five broad approaches. The first approach focuses on decreasing the immunogenic content of gluten, using strategies like genetically modified wheat, intra-intestinal gluten digestion using glutenases, microwave thermal treatment of hydrated wheat kernels, and gluten pretreatment with either bacterial/ fungal derived endopeptidases or microbial transglutaminase. The second approach involves sequestering gluten in the gut lumen before it is digested into immunogenic peptides and absorbed, using binder drugs like polymer p(HEMA-co-SS), single chain fragment variable (scFv), and anti- gluten antibody AGY. The third approach aims to prevent uptake of digested gluten through intestinal epithelial tight junctions, using a zonulin antagonist. The fourth approach involves tissue transglutaminase (tTG) inhibitors to prevent the enhancement of immunogenicity of digested gluten by the intestinal tTG enzyme. The fifth approach seeks to prevent downstream immune activation after uptake of gluten immunogenic peptides through the intestinal mucosal epithelial layer. Examples include HLA-DQ2 blockers that prevent presentation of gluten derived- antigens by dendritic cells to T cells, immune- tolerizing therapies like the vaccine Nexvax2 and TIMP-Glia, cathepsin inhibitors, immunosuppressants like corticosteroids, azathioprine etc., and anti-cytokine agents targeting TNF-α and interleukin-15. Apart from these approaches, research is being done to evaluate the effectiveness of probiotics/prebiotics, helminth therapy using *Necator americanus*, low FODMAP diet, and pancreatic enzyme supplementation in CeD symptom control; however, the mechanisms by which they play a beneficial role in CeD are yet to be clearly established. Overall, although many therapies being explored are still in the pre-clinical phase, some like the zonulin antagonist, immune tolerizing therapies and glutenases have reached phase II/III clinical trials. While these potential options appear exciting, currently they may at best be used to supplement rather than supplant the GFD.

Keywords: prolyl endopeptidase (PEP), glucocorticoids, exocrine pancreatic insufficiency, immune tolerogenesis, gluten, genetically modified wheat, helminth therapy, larazotide acetate

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INTRODUCTION

Celiac disease (CeD) affects 0.7% of the global population (1). Although initially believed to affect only the intestine, it is now considered to be a systemic autoimmune disease. The consequent spectrum of manifestations of CeD is wide, ranging from gastrointestinal and nutritional derangements resulting from the enteropathy to neuropsychiatric symptoms, infertility and liver diseases, among extra-intestinal manifestations (2). For the affected individuals (3) and their care providers (4, 5), it can adversely affect the quality of life.

Since the discovery of dietary gluten as the causative agent six decades ago (6), our comprehension of the pathophysiology of CeD has grown substantially. This process has been facilitated by documented clinical observations as well as exponential advancements in immunology and molecular medicine. However, the cornerstone of management of CeD is based on the lesson learnt from that discovery itself, and it still remains the gluten- free diet (GFD). Patients may find the GFD and related lifestyle modification to be burdensome, due to its poor satisfactoriness, unavailability and higher costs (7). In fact, hyper- vigilance to GFD adherence can also affect quality of life (8), much like the disease itself. This has in turn created an unmet need for alternatives (9). Also, there may be patients who remain persistently symptomatic despite a GFD, in whom adjunct therapies may be required (10). Herein, we have reviewed the principles of potential, alternative dietary and non-dietary therapeutic strategies and their current status of investigation (**Supplementary Table 1**).

PATHOGENESIS

In a broad sense, the pathogenesis of CeD may be considered to be a result of the interplay of genetic susceptibility and immunological factors, described as follows.

Genetic Basis

CeD is caused by autoimmunity to gluten in genetically predisposed individuals, with frequently observed familial clustering (11). The main implicated alleles are the HLA-DQ2 and HLA-DQ8, that contribute to 30–50% of genetic susceptibility (12). Genotyping studies have also identified non-HLA variants associated with CeD, with most genes in these variants involved in the structure or function of immune cells (13–16). However, the non-HLA genes carry only a modest increase in the risk of CeD (17). Regardless, all these genes represent potential targets for therapeutics. Some of them, along with their respective loci are summarized in **Table 1**.

TABLE 1 | Common gene loci involved in celiac disease.

Locus	Gene	Chromosome	References
CELIAC1	HLA-DQ2 and HLA-DQ8	6p21.3	(15)
CELIAC2		5q31-33	(14)
CELIAC3	Intergenic between CD28, CTLA4 and ICOS	2q33	(16)
CELIAC4	MYO9XB (myosin IXB gene)	19p13.1	(13)

Immunogenicity of Gluten

Gluten is found in wheat as well as barley, rye and oats. Gluten is composed of two peptide conglomerates viz. glutelins and prolamins (18). Of these, prolamins are the gluten components that are implicated in CeD; they are found variously in different grains as gliadins in wheat, secalins in rye, a mix of both in triticale, hordeins in barley, and avenins in oats. In wheat, gliadins are in turn composed of sub-fractions–α/β, γ, ω1, ω2, and ω5 (**Figure 1**). The α-gliadin sub-fraction has the maximum immunogenicity in CeD, contributing the most to toxic epitopes upon digestion (19).

At the molecular level, gliadins are made predominantly of multiple glutamine (35%) amino acid residues linked to proline (15%) (20). Presence of proline makes the structure of prolamins complex as well as sterically inaccessible/ resistant to proteolytic enzymes of the human stomach and intestine. Consequently, these luminal proteases only succeed in digesting the prolamins into larger oligopeptides. These oligopeptides are immunogenic in CeD (21) and some of them include, the most immunotoxic, 33- mer peptide 57–89 (with the amino acid sequence LQLQPFPQPQLPYPQPLPYPQPQLPYPQPQPF), and relatively less immunogenic ones like peptide 57–73, peptide 111–130, 26- mer peptide with the sequence FLQPQQPFPQQPQQPYPQQPQQPFPQ), peptide 31-43 etc. (22–27).

The digested gliadin peptides enter the lamina propria of the small intestine across the epithelial barrier, by a paracellular pathway that involves the protein zonulin (**Figure 2**). Zonulin is structurally similar to the zona occludens toxin associated with *Vibrio cholera* and has been observed to be a controller of epithelial permeability. In the zonulin pathway, gliadin products attach to the chemokine receptor CXCR3 on the luminal aspect of the intestinal epithelium. CXCR3 in turn increases the formation of zonulin, which relaxes the inter-epithelial tight junctions through the PAR2/EGFR (Protease activated receptor 2/Epithelial Growth Factor Receptor) pathway. This increased permeability leads to influx of gliadin (28). An alternative pathway implicated in gliadin uptake is the transcellular pathway. This involves secretory Immunoglobulin A (IgA) that co-localizes with another molecule, the CD71 to promote transcellular uptake of gliadin products into the lamina propria (29). CD71 is the transferrin receptor, but is found to be expressed in higher amounts on the luminal aspect of intestinal epithelial cells in CeD.

The tissue transglutaminase-2 (tTG-2) enzyme modifies the digested gluten immunogenic peptides that have entered the mucosa, by deamidating their glutamine residues to glutamate. These negatively charged glutamate side chains have a higher potential to be recognized as immunogenic. Also, by virtue of the relatively large size of these partially digested proline containing fragments, and the negative charge of glutamate, they tend to settle and form bonds with the neighboring extracellular matrix, resulting in immobilized neoepitopes. The formation of these bonds may be directly catalyzed by the tTG

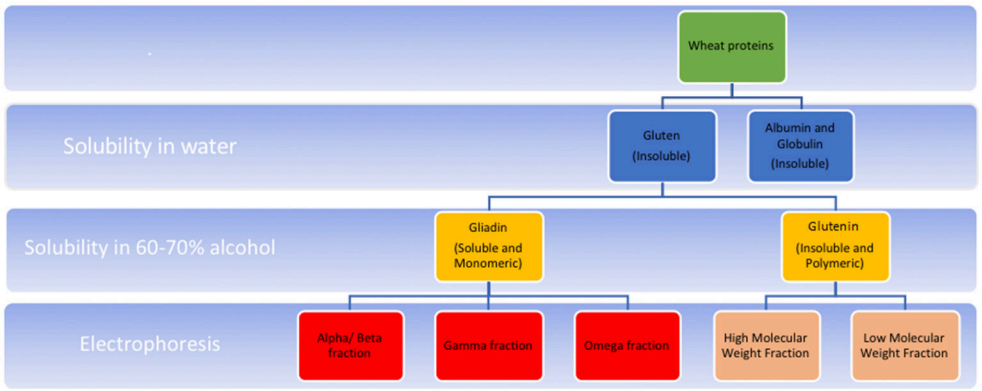

FIGURE 1 | Schematic representation of composition of wheat gluten. Gluten refers to the water insoluble protein component left after washing wheat flour. In various cereals, gluten is a conglomerate of peptides, composed of two main fractions-prolamins (known as gliadins in wheat), and glutelins (known as glutenins in wheat). These fractions differ in their solubility in alcohol; the former of these is alcohol soluble. Also, prolamins occur in monomeric form with intrachain disulphide bridges formed by cysteine residues. Glutelins, in contrast, occur as polymers formed by interchain disulphide bridges in addition to intrachain bridges. Gliadin is composed of further subfractions-α/β, γ (each having intrachain disulphide bridges) and ω (having no disulphide bridges) which differ in their electrophoretic mobilities in a low pH medium. Glutenin fraction, upon reduction of the interchain bridges, in turn yield high molecular weight (HMW), and low molecular weight (LMW) fractions depending on their mobility on SDS PAGE electrophoresis. High molecular weight fractions of glutelin have less disulphide bonds compared to the low molecular weight fractions.

(30). Ultimately, all the gluten- derived antigens are recognized and processed by the HLA-DQ2 and -DQ8 bearing antigen presenting cells (APCs), which activate CD4+ helper T cells, setting off an inflammatory cascade. Activated CD4+ cells release cytokines like Interferon- γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α), thereby further enhancing the permeability and facilitating a self-propagating mechanism of gliadin uptake. T-cells also activate B-cells which mature to produce antibodies against gluten and tissue transglutaminase-2 (celiac antibodies). These antibodies further contribute to the ensuing immune-mediated enteropathy. As well, the immunotoxicity is mediated through the increased production of interleukin-15 (IL-15) by the intestinal epithelial cells and the intraepithelial lymphocytes (IEL). IL-15 upregulates the receptor Natural Killer Group 2D (NKG2D) expression on IEL which interacts with MHC class I polypeptide-related sequence A and B (MIC-A and MIC-B) ligands displayed on epithelial cells (31). IELs also express an NK receptor called CD94/NKG2C. CD94/NKG2C recognizes Human Leukocyte Antigen-E (HLA-E), a protein that is upregulated in epithelial cells in response to IFN-γ in CeD (32). The interaction of these two ligand-receptor pairs activates the IELs and triggers them to kill epithelial cells through perforins, and other mechanisms (33).

NOVEL THERAPIES FOR CELIAC DISEASE

Our understanding of the pathogenetic pathway of CeD has provided the context for the development of new drugs that target different aspects of this pathway. An overview of the mechanisms of action of these drugs is given in **Figure 2**. The therapeutic options for CeD (**Supplementary Table 1**) currently being investigated can be broadly classified as belonging to one of the following approaches.

Approach 1: Decreasing Immunogenic Epitopes in the Gluten

Genetically Modified Wheat

Bread wheat (*Triticum aestivum*) has a hexaploid genome AABBDD, wherein chromosomes 1 and 6 predominantly harbor the genes known to code for immunotoxic components of gluten (34). Attempts have been made to manipulate these genes to attenuate immunotoxicity. However, doing so may also alter the gastronomic properties of wheat, the yield etc, if these properties are governed by the same or neighboring genetic loci. Although studies have explored the genetic manipulation of these chromosomes, a variant that is both safe in CeD and suitable for commercial production is yet to be found.

A study explored a variant formed by the removal of genes on chromosome 1 that code for β, γ, and ω gliadin fractions. While the toxicity was attenuated, the mechanical properties of wheat were not altered. However, when α fraction was attenuated instead, the mechanical properties were compromised while also significantly reducing the dose of immunogenic T cell epitopes (35).

Prolamins from a wheat variant called C173 were tested *in vitro* on intestinal epithelial cells derived from CeD patients. This variant was formed by the removal of predominantly toxic epitopes in gliadin fractions viz. Gli-A2, Gli-D1, and Gli-D3. There was no worsening of villous: crypt (V:C) ratio, however there was an increase in pro- inflammatory cytokines like IFN-γ, TNF-α as well as in anti- tTG antibody levels in the collected supernatant (36). Therefore, this was unlikely to be suitable for further clinical testing in CeD.

In another recent development, the International Wheat Genome Sequencing Consortium delivered a high-quality annotated reference genome sequence of the Chinese spring wheat (37). The sequence is referred to as the RefSeq v1.0. The process started in 2005 and has now yielded the sequence of

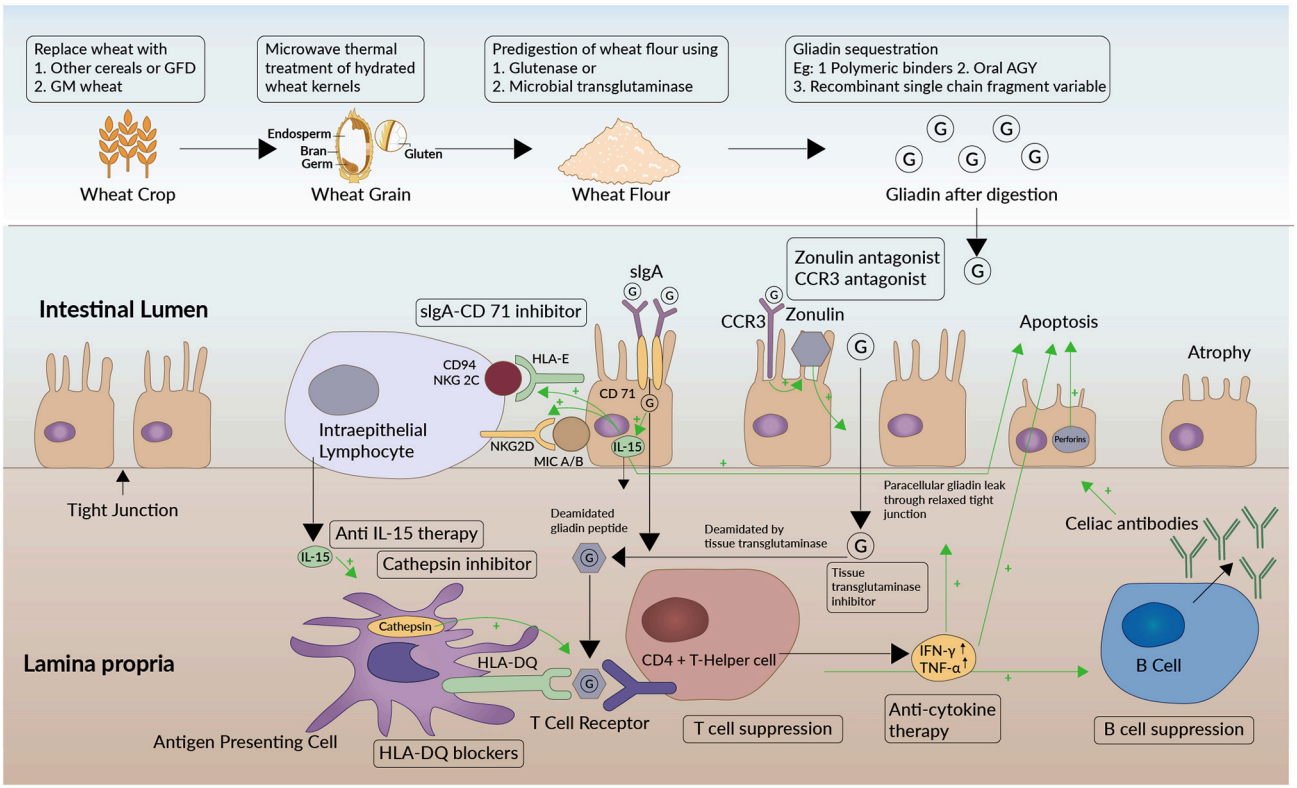

FIGURE 2 | Target sites of therapeutics along the pathogenetic pathway of celiac disease. Sites of action of therapeutic approaches under investigation (enclosed in black boxes) are shown at different levels of the pathogenetic pathway of celiac disease. Green arrows in the figure depict a stimulatory effect. The oligomers (G) formed from gluten digestion enter the lamina propria of the small intestine across the epithelial barrier. They do so by a paracellular pathway that involves the protein zonulin. Zonulin is structurally similar to the zona occludens toxin expressed by *Vibrio cholera* and regulates epithelial permeability at apical tight junctions. In the zonulin pathway, gluten products attach to the chemokine receptor CXCR3 on the luminal aspect of the intestinal epithelium that increases the formation of zonulin. The zonulin then relaxes the interepithelial tight junctions through the PAR2/EGFR (Protease activated receptor 2/Epithelial Growth Factor Receptor) pathway. This increased permeability in turn leads to influx of gliadin. An alternative pathway implicated in gliadin uptake is the transcellular pathway involving secretory IgA (Immunoglobluin A) and CD71. CD71 or transferrin receptor is found to be expressed in higher amounts on the luminal aspect of intestinal epithelial cells in CeD. The CD71 co- localizes with secretory IgA and has been postulated to promote transcellular gliadin uptake into the lamina propria in CeD. Zonulin antagonists, CXCR3 antagonists, and sIgA/CD71 pathway antagonists would prevent gliadin transport through either of these two pathways. Once the gluten immunogenic epitopes are transferred into the lamina propria, the HLA-DQ2 and -DQ8 bearing antigen presenting cells (APCs) recognize the epitopes. Consequently, APCs activate CD4+ helper T cells, setting off an inflammatory cascade. Cathepsins play a role in processing the antigens in APCs and promoting the interaction between APCs and CD4+ T cells. Cytokines like IFN-γ and TNF-α are released by activated CD4+ cells, which further aggravate this permeability and facilitate a self-propagating mechanism. T-cells also activate B-cells which mature to produce antibodies against gluten and tissue transglutaminase−2 (celiac antibodies). HLA-DQ blockers, anti-cytokine therapy, and cathepsin inhibitors are some of the therapeutic approaches being explored. In addition, the tissue transglutaminase-2 (tTG-2) enzyme deamidates the glutamine residues to glutamate in gliadin. The resulting deamidated gliadin peptides are more immunogenic. Also, by virtue of the relatively large size of these partially digested, negatively charged proline containing fragments, they tend to settle and form bonds with neighboring tissues resulting in immobilized neoepitopes. Thus, these peptides form immunoreactive autoantigens and enteropathy ensues. tTg-2 enzyme inhibitors are being explored as therapeutic options. The immunotoxicity is mediated by the increased production of interleukin-15 (IL-15) by the intestinal epithelial cells as well as by the intraepithelial lymphocytes (IEL) in CeD. IL-15 upregulates the receptor NKG2D (Natural Killer Group) expression on IEL which interacts with MIC-A and MIC-B (MHC class I polypeptide-related sequence A and B) displayed on epithelial cells. IELs in patients with CeD also express an NK receptor called CD94/NKG2C. CD94/NKG2C recognizes HLA-E, a protein that is upregulated in epithelial cells in response to Interferon- γ (IFN-γ). The interaction of these two ligand- receptor pairs activates the IELs and triggers them to kill epithelial cells through perforins. Among other therapeutic options, gluten from wheat may be rendered non-immunogenic by genetic modification. Attenuation of immunotoxicity has also been attempted using microwave energy application on hydrated wheat kernels or by gluten modification either using glutenases from fungi or bacteria or using microbial transglutaminase. Alternatively, gliadin in gluten may be sequestered in the intestinal lumen using polymeric binders, AGY or oral Immunoglobulin Y, and recombinant single chain Fragment variable.

107,891 high-confidence genes, including the genomic context of their regulatory sequences. This has the potential to fast track development of genetically engineered wheat with attenuated immunotoxicity while preserving its gastronomic or agronomic properties (38).

Intraluminal Digestion of Gluten Using Oral Glutenases

In order for gliadin-derived oligomers to enter the lamina propria and still not induce an immune reaction, the oligomers should contain nine amino acids or less (39). Therefore, gluten

degrading enzymes (glutenases) that digest gliadin into peptides with nine or less amino acid moieties have been explored as a therapeutic option in CeD. Most of these are glutamine and proline specific enzymes, since these are the principal amino acids found in immunogenic motifs of gluten. The forthcoming sections will highlight some of these enzymes.

The glutenase EP-B2 (endoprotease B, isoform 2) is a glutamine specific peptidase. Being a cysteine protease enzyme, it has a Cys-His-Asn catalytic triad in its active site. It is secreted naturally in the acidic endosperm of germinating barley seeds (*Hordeum vulgare*) where it serves to digest hordein, the analog of gliadin. Glutenase EP-B2 is optimally active at low pH, resistant to pepsin but lysed at physiological concentrations of trypsin, and has good specificity for the sequence QXP, which is abundant in the 33-mer as well as other immunotoxic gluten sequences. These factors make it a good fit for therapy in CeD as a gastric active enzyme (40). Gass et al. studied its action in animal models. They observed the complete digestion and liquefaction of a gluten containing meal into a viscous fluid in gliadin-sensitized Wistar rats that were fed this enzyme along with the meal. The stomachs of the rats that did not get the enzyme contained a relatively solid, dry paste-like material post digestion of the same meal. This effect was even more evident at higher doses and extended digestion times (41). One of the concerns with any gastric-based glutenase is that if the gastric emptying occurs before the complete digestion of gluten, immunotoxic residue would reach the duodenum and induce the disease. It is therefore reasonable to combine this enzyme with other glutenases which are stable in the gastric environment but start acting when the food chyme reaches duodenum, preempting any immunotoxicity in the intestinal epithelium.

Proline specific endoproteases (PEP) from the microbes *Flavobacterium meningosepticum* (FM-PEP), *Sphingomonas capsulata* (SC-PEP), and *Myxococcus xanthus* (MX-PEP) have also been investigated as potential glutenases. They are serine proteases and each has a larger β-propeller domain and a smaller, N-terminal catalytic domain that breaks the peptide bond of proline residues at the carboxy end of the gluten protein (42). Activity of SC-PEP extends into the acidic range of pH, and is by and large, unaltered in the presence of pepsin (43). However, the other two PEPs are lysed by pepsin. Furthermore, FM-PEP is inactivated by the small intestinal enzyme trypsin in the presence of bile acids. MX-PEP too is unstable in the presence of bile salts (44). Considering these limitations, SC-PEP has been explored as a more favorable candidate for therapy in CeD. In order to improve its action further, mutant variants (variant 10,224 or 10,230) of SC-PEP have been developed which have 200-fold higher resistance to pepsin and 20% higher turnover at acidic pH (45).

While SC-PEP has high specificity for gluten immunogenic epitopes, it has relatively low specificity for long peptide sequences. This is because the larger β-propeller domain preferentially allows smaller gliadin fragments into the active site, and therefore, is unable to completely eliminate the immunogenic gliadin peptides. This limitation could be overcome by combining it with other enzymes with complementary specificity. Combination of EP-B2 with SC-PEP, for instance has been explored for application in CeD. The EP-B2 efficiently digests the 33 mer peptides into smaller, not necessarily non-toxic proline containing fragments. The PEP complements its action by digesting the proline- glutamine links in these smaller oligopeptides, thereby reducing their immunotoxicity. Gass tested this combination on rat models and found that 1:1 ratio of this oral enzyme combination was more efficacious than either enzyme alone in reducing immunotoxic oligopeptides from gliadin (46).

Several clinical trials have been conducted to assess the effectiveness of this enzyme mixture in making dietary gluten safe for patients with CeD. One of the most prominent of these has been using the enzyme cocktail ALV 003, now known as latiglutenase. Latiglutenase is a proprietary, 1:1 combination of EP-B2, or ALV 001 plus PEP, or ALV 002. A randomized control trial (RCT) studied CeD patients on GFD, who received a diet containing gluten (16 g/day for 3 days) pre-treated with either ALV003 or placebo (NCT00859391). The ALV003 group showed significantly lower immunological activation, as seen on peripheral T cell IFN-γ responses to gliadin (47). A phase 2a RCT (NCT00959114) on 41 CeD patients on GFD, found that on gluten challenge (with 2 g bread crumb for 6 weeks), V:C ratio deteriorated significantly less in patients treated with ALV003 than with placebo. However, intraepithelial $CD3^+$ lymphocytes remained unchanged in the ALV003-treated patients compared to placebo-treated patients. This study was also significant for having used bread crumbs to simulate real life gluten intake as opposed to pre-digested gluten (48). Although the aforementioned clinical trials were promising, the results of the most recent large-scale phase 2 b trial (NCT01917630) were disappointing. Four hundred ninety CeD patients who were symptomatic despite GFD for a year, were included in a dose ranging, placebo-controlled double-blinded study. In a modified intention-to-treat analysis, no significant differences were observed on histological, serological or symptomatic end-points (49). In a *post-hoc* analysis of data, it was found that there was significant reduction in the abdominal symptoms in those patients who were seropositive for celiac antibody. The authors concluded that seronegative patients did not experience any symptomatic improvement, possibly because their symptoms may be attributable to non-celiac causes (50). Another phase 2 study is underway to test the effect of ALV003 on histopathological parameters of 80 CeD patients who had been adherent to a GFD (NCT03585478). Overall, therefore, the effectiveness of ALV003 remains to be established.

Kuma030 is an engineered glutenase developed by the Institute for Protein Design, University of Washington. Prior to its development, the investigators identified a naturally occurring enzyme from the acidophilic microbe Alicyclobacillus sendaiensis, called kumamolisin-As (KumaWT). It is a serine endoprotease, with optimal activity over the pH range of 2–4/37°C and therefore adaptable for use in the gastric environment (51). Based on the structure of KumaWT, an enzyme was designed, with specificity toward known gliadin peptides. The new enzyme called KumaMax or Kuma010, had 116 times higher proteolytic activity, and 877 times higher specificity for the target gliadin oligopeptides. This enzyme was

further modified to result in several-fold higher activity against immunogenic 33-mer and 26-mer peptides. This version was called Kuma030. In comparison to SC PEP- EP B2, Kuma030 seemed to be more efficient. At the highest concentration of the PEP-EP B2 i.e. 1:10 weight/weight (w/w) ratio, gluten was degraded by 84.4%. Kuma030 at a lesser concentration of 1:40 w/w ratio achieved >99.9% gliadin degradation thereby reducing the gluten content to 3 ppm, well-below the 20 ppm threshold for "gluten-free" labeling. When gluten sensitive T cells isolated from patients with CeD were incubated with gliadin pre-treated with Kuma030, a dose dependent reduction in IFN-γ production and T cell proliferation was observed (52). In light of these findings Kuma030 may hold promise in the near future.

AN-PEP (*Aspergillus niger*- Prolyl Endopeptidase), like SC-PEP, is a glutenase with optimum activity at gastric pH between 3 and 5. It is also similarly resistant to proteolysis by pepsin, but has 60 times faster action compared to another prolyl endopeptidase such as MX-PEP (53). *A.niger*, the fungus from which this enzyme is derived is a food grade microbe, available on an industrial scale. AN-PEP has therefore been explored for suitability as an oral supplement. Being a relatively non-specific enzyme, it has less specificity for immunogenic epitopes on gluten. However, it is highly efficient in degrading gliadin into smaller peptides, and may potentiate action of more specific enzymes if used in combination. It is currently in Phase 2 clinical trials (54). In preliminary studies, the enzyme has been found to catalyze almost complete degradation of gluten epitopes even in complex food matrices such as in fast food meal. Also, co-administration of AN-PEP with gluten to CeD patients led to a complete elimination of T-cell stimulatory peptides from both gliadins and glutenins within 2 h, as measured in their gastric aspirates (55, 56). Another trial (NCT00810654) randomized CeD patients to receive 7 g of gluten along with either placebo or AN-PEP for 14 days. No significant difference in serology, symptoms, or histopathology was observed between the groups, with no patient in either group showing any significant deterioration. However, mucosal IgA tTG deposits were observed in four patients in the placebo group compared to 1 in the AN-PEP group, showing some mitigation of serological response in the latter (57). Trials with bigger samples and longer follow up are warranted for conclusive results on the therapeutic value of the enzyme. Tolerase G is a commercially available form of this enzyme.

Dipeptidyl peptidase- IV (DPP-IV) is an exopeptidase that acts on the amino-terminal side to liberate X-Pro dipeptides in gliadin. It occurs naturally in small amounts in the small intestinal brush border. It has been obtained commercially from the fungus *Aspergillus oryzae* and its potential as a glutenase has been investigated. On its own, DPP-IV has modest efficiency as it can only act on peptides starting with X-Pro. Additionally, DPP-IV has a neutral pH optimum and hence it starts action only in the intestine. Addition of AN-PEP to DPP-IV may however improve its efficiency. The combination when administered as an oral mixture has been found to successfully degrade small amounts of gluten (54). Because of non-specificity of AN-PEP and the very limited proteolytic effect of DPP-IV, the effect of this combination appears to be modest, at best. This combination was studied as a part of STAN1, a cocktail of microbial enzymes commonly used in food supplements. A RCT was done on 35 CeD patients that were persistently seropositive despite GFD. They were randomized to receive either STAN1 or placebo, along with 1 g of gluten per day for a total of 12 weeks. This study found no difference in serology between the two arms (58).

Triticain α, from the papain family, is a cysteine protease and therefore optimally active in the acidic pH. This enzyme possesses collagenase and glutenase activity and occurs naturally as a zymogenic precursor in *Triticum aestivum* (bread wheat). In a gastric like milieu, it exhibits relative resistance to pepsin cleavage, as well as optimal activity at a pH of 3.0/37°C. Upon incubation with gluten, it causes the cleavage of α, γ, ω, and glutenin fractions. However, in presence of trypsin/pH 8.0/37°C, the enzyme is susceptible to destruction. Hence it may be effective only in the gastric digestion of wheat gluten prior to the food bolus reaching the intestine (59). Further *in vitro* testing to study its efficiency as detoxifying enzyme in celiac disease is required. Caricain, another cysteine protease/ prolyl endopeptidase preparation (EC 3.4.22.30) is a derivative of papaya. In a RCT, upon co-administration of caricain with 1 g gluten daily to 20 CeD patients in remission, patients had no worsening of symptom scores and histopathology (60). It is available as a supplement called Gluteguard. Again, more studies would be required to determine its utility in CeD.

Also in nascent stages of investigation are other potential glutenases like the enzyme Pseudolysin (IasB) from *Pseudomonas aeruginosa* isolated from human fecal microbiota (61), the enzyme Nepenthesin from pitcher plants (62) and an unknown enzyme from human salivary plaques (63). In addition to these, there has been a surge of commercially available dietary supplements that claim to aid the digestion of gluten and they are marketed to patients with CeD or to those who prefer to restrict dietary gluten for other reasons. These products are mainly glutenases like DPP-IV, which is known to have limited proteolytic activity. Some of their potential adverse effects, apart from worsening of CeD, include allergy to other components used like egg. None of these products are currently FDA approved (64).

Although all the enzymes described in the preceding sections have been proven glutenase activity under appropriate thermochemical conditions, whether these enzymes completely eliminate all immunogenic epitopes and prevent any possible immune-activation by dietary gluten is the most pertinent question. Moreover, most of the glutenases that are now in advanced stages of clinical trials, have been studied in the context of small amounts of gluten challenge, in patients who are already on the GFD. Such small challenges simulate inadvertent gluten exposure in patients that adhere to GFD, and are useful to study patients that are not responsive despite adherence to GFD. However, the patients who should ideally benefit from these medications are those that find it difficult to adhere to strict GFD or would like to consume near normal levels of gluten in their diet. Whether oral glutenases would help them remains to be proven. Clinical trials with higher doses of gluten challenge are required.

Enzymatically Modified Wheat Gluten

Gluten modification using glutenases.

Wheat flour can be modified by fermentation with bacteria or fungi. These organisms release proteolytic enzymes that digest gluten, rendering it less toxic, although with modified texture of the flour. This flour can then be mixed with other flours like buckwheat, millets, amaranth etc. to restore its viscoelastic properties.

In a study, sourdough lactobacilli were used to ferment wheat. These organisms release proteases that digest the highly immunogenic 33 mer peptide from gliadin. The species used were *L. alimentarius, L. brevis*, *L. sanfranciscensis,* and *L. hilgardii*. They were selected previously on the basis of the hydrolytic activities of their enzymes iminopeptidase, dipeptidyl-peptidase prolyl endopeptidase, prolidase, prolinase, and aminopeptidase that are known to digest gluten (65). These bacilli were added to sourdough flour made with wheat (30%), non-toxic oats, millet, and buckwheat and fermented for a day. The impact of ingestion of bread made from this flour on intestinal permeability was assessed in CeD patients using the lactulose/rhamnose excretion ratio. The intestinal permeability was found to remain unchanged as compared to the baseline (66). In another study, feeding a diet of baked goods (8 g of gluten) made from a similar fermented wheat flour, to CeD patients, did not show any impact on the serological, histological, and immunohistochemical parameters (67). Further studies would be required to prove the safety of fermented wheat flour in CeD patients.

A few studies have tried to address the issue of palatability of fermented wheat by adding other flours. Heredia-Sandoval et al. showed that addition of Amaranth flour to enzymatically pre-digested wheat was found to render it acceptable with good viscoelastic properties and at the same time less immune-reactive. The enzyme used in this study was the glutenase AN- PEP (68).

Gluten modification using microbial transglutaminase.

Another enzymatic approach that has been investigated is the use of microbial transglutaminase (mTG), isolated from *Streptoverticillium mobaraensis* (69). It is a food grade enzyme and is already extensively used in the food industry to improve the mechanico-chemical properties of food. Unlike the proteolytic enzymes discussed in the preceding sections, it crosslinks gluten molecules in the presence of an amine donor like lysine or lysine methyl ester. It has the same site specificity as the human tTG, but in contrast, lacks deamidase activity, and is not dependent on calcium (70). The enzymatic action of mTG has been hypothesized to attenuate the gluten immunogenic epitopes. Some *in vitro* studies (71), animal studies and *in vivo* studies in the intestinal explants from patients with CeD (72) have suggested reduction of immunogenicity of gliadin in mTG modified wheat flour. In a phase two clinical trial, seven patients each with CeD in remission were fed 100 g of either enzyme modified wheat rusks or modified wheat alone for 90 days. Less number of patients showed elevation in celiac antibodies (2 vs. 4) and worsening of villous abnormalities (1 vs. 4) in those who were fed modified wheat compared to those fed unmodified wheat (69).

Another set of studies have attempted to investigate if the transamidated gluten peptide end products of this enzyme have any immunotoxicity similar to the products of human tTG. For example, Yong et al. showed that the enzyme increases the deamidated end products by 70% when allowed to act on gluten at 40°C and neutral pH (73). Another study found similar results as well (74). Hence, overall, the safety of microbial transglutaminase in CeD is unclear.

Thermally Modified Wheat Gluten-Gluten Friendly Bread

Di Luccia et al. (75) developed a technology to detoxify wheat gluten proteins using microwaves (76). Prior to milling, microwave energy is applied for a few seconds to cleaned, hydrated wheat kernels at 15–18% humidity, to reach a high temperature within a short period of time. The process is repeated over several cycles until a temperature of 80–90°C and moisture of 13–13.5% in the grains is reached. After this, grains are dried over 24 h at room temperature and milled. This process had been proposed to attenuate the immunotoxicity of gluten by 99%, as detected by the R5 monoclonal antibody method, which is a method of detection of gluten immunogenic peptides (77). The bread from this flour was called "gluten friendly or GLUFR." However, a later study found the immunotoxicity of this flour to be unchanged, when checked by the G12 method (another antibody-based gluten immunogenic detection test), mass spectrometry-based proteomics and *in vitro* assay with T cells of celiac subjects (78). It is possible that microwave therapy causes reconfiguration of the gluten structure that interferes with detection of gluten immunogenic peptides by R5 ELISA method. A clinical trial (NCT03137862) evaluating the safety and efficacy of 3 vs. 6 grams of gluten friendly bread for 12 weeks in CeD patients in remission has also been completed, the results however are yet to be published. The results of another *in vivo* study (NCT03168490) evaluating the effect of GLUFR flour on intestinal microbiome and symptoms of patients remain to be seen.

Approach 2: Intraluminal Sequestration of Gluten Immunogenic Epitopes

Polymeric Binders

Polymeric binders are used in certain diseases to sequester toxic compounds in the gastrointestinal tract (79). They have also found application in CeD, where they act by binding gluten in the gut and preventing their breakdown/ absorption. One example is poly (hydroxyethyl methacrylate-*co*-styrene sulfonate) or P(HEMA-*co*-SS), a non-absorbable, high molecular weight, linear co-polymer of hydroxyethylmethacrylate (HEMA), and sodium 4-styrene sulfonate (SS). The experimental form of P(HEMA-*co*-SS), called polymer BL-7010, was studied by Liang et al. They demonstrated the mechanism of the sequestration of alpha gliadin by this polymer using spectroscopic and light scattering methods (80). This was followed by *in vitro* studies that demonstrated no toxic effect of gliadin on cell permeability in the presence of the polymer (81). Subsequent *in vivo* studies using HLA-HCD4/DQ8 mice revealed selectivity in sequestration

of gliadin and hordein as compared to other nutrients, as well as reduction in the villous damage caused by the gluten (82).

In order to test practical applicability of industrial grade preparation of the polymer, McCarville and colleagues studied the effectiveness of two batches of BL-7010 together, original polymer A and the industrial preparation polymer B. The latter is structurally similar to the original polymer described by Pinier et al. (81) but has better yield during scaled up industrial preparation. Additional aims were to determine the binding specificity with gliadin and with nutrients, to evaluate the genetic toxicity *in vitro* and the safety and systemic absorption of unlabeled BL-7,010 as part of a toxicology study in mice. For the effectiveness study they prepared transgenic mice deficient in MHC II, expressing HLA-DQ8. They were initially bred on gluten free diet. They were treated with anti-CD25 antibodies to deplete CD4+CD25+Foxp3 cells, which mediate immune tolerance. Then at age 6 weeks onwards they were sensitized with pepsin trypsin digested gliadin and cholera toxin (CT). Post sensitization, mice were administered gluten plus either polymer A or polymer B. It was observed that the polymers bind avidly to the gliadin, with no interaction with vitamins, pepsin, and pancreatin and minimal interaction with albumin. Moreover, systemic absorption was negligible and repeated toxicity studies showed safety in the mice model. While polymer A was completely effective in abrogating villous damage, polymer B was slightly less effective (83).

Phase 1 human studies were completed in October 2014 (NCT01990885), the results of which are yet to be published. The trial involved 40 celiac adult patients who were well-controlled after 6 months of adherence to GFD, and had negative IgA-EMA and IgA-anti tTG antibodies. Escalating, repeated doses of BL-7010 were administered in addition to gluten in a cross over, placebo controlled RCT. The outcomes planned to be assessed were adverse events and safety parameters, as well as plasma levels of BL-7010.

Polymeric binders may be useful to treat inadvertent or minimal gluten exposure and have a potential role, at the very least, as supportive therapy in addition to GFD. Results of human trials with dose variations will throw more light on the extent to which they may be helpful.

Anti-gluten Antibody

Chicken egg yolk may be used to produce antibodies to confer passive immunization. IgY is an antibody harvested from the yolk of eggs laid by hens that have been super immunized against gliadin. Gujral et al. showed that these antibodies can be made to significantly neutralize gliadin fraction, in gut- like *in vitro* conditions. They also demonstrated that these IgY antibodies can be put into a capsule form with 50% mannitol. This encapsulated form is called AGY, and it is resistant to degradation in the acidic pH of the stomach. *In vitro* testing showed that gliadin absorption was decreased from 42.8 to 0.7% with the addition of AGY. Also, it was found to be more effective in the presence of food (84). AGY was subsequently tested in a phase one trial to check safety as well as efficacy in improving symptoms of CeD patients who were persistently symptomatic despite adhering to a GFD. AGY capsules were administered along with meals for 6 weeks to the patients. Ten patients completed the study, and no safety concerns were identified. Most patients had fewer celiac symptoms, improved quality of life, lowered antibodies, and lowered lactulose mannitol ratio when taking AGY as compared to the baseline run-in period (85).

The initial results seem encouraging; although testing on a bigger sample size would be required to confirm its benefits. It has minimal toxicity, when administered orally to humans as it does not get absorbed into the systemic circulation to cause systemic immune activation. However, egg allergy would be a contraindication for its use.

Single Chain Fragment Variable

Fab is the antigen binding site of an antibody fragment. This Single chain fragment variable (scFv) is a fusion protein that contains the Fab sites of the variable and light chains of the antibody. scFv has been explored for therapeutic use in cancer immunotherapy. It is also being tested *in vitro* for its effectiveness in neutralizing gliadin, for celiac therapy (86). The initial step of the antibody fragment production requires invoking an immune reaction in chicken with gliadin. The birds then serve as a source of RNA with the sequence for the scFv (87). This scFv sequence is subsequently used to produce recombinant scFV in *E. coli* bacteria in larger amounts. Since two antigen binding regions increase binding affinity, two scFv may be joined with a peptide linker to create a tandem scFv (tscFv). This tandem fragment was tested by Eggenreich et al. who found that scFv had the highest affinity of binding to digested gliadin, followed by wheat, and spelt flour. There was no binding with rice and millet flours, indication specificity of binding (86). In order to test this fragment in human subjects, gastric resistant preparations would need to be produced.

Approach 3: Prevention of Uptake of Gliadin Epitopes

Tight junctions are apical, intercellular junctions that regulate the passage of molecules via the paracellular transport pathway. In normal conditions, pathogenic bacteria, and dietary antigens are prevented from passing through the tight junctions. Changes in the paracellular permeability have been hypothesized to be an early event in the pathogenesis of CeD (88). Immunostimulatory gluten peptides then pass through the paracellular route in such individuals. Human protein zonulin, has been found to be a regulator of epithelial permeability and is highly expressed in the mucosa and blood of patients with celiac disease. It is similar to the zonula occludens toxin (ZOT) expressed by Vibrio cholerae, which impairs integrity of epithelial tight junctions. Gliadin binds to the chemokine receptor CXCR3 releasing Zonulin which subsequently increases the intestinal permeability via the MyD88 dependent pathway (28).

The transcellular gliadin transport pathway can be exploited for management of CeD. Larazotide acetate, a drug targeting Zonulin has been developed by Alba therapeutics, a biopharmaceutical company in USA. Formerly referred to as AT-1001, it is an octapeptide that antagonizes zonulin. Larazotide was found to cause promotion of assembly of actin and E- cadherin around tight junctions of Madin-Darby canine

kidney (MDCK) type II cells, thus promoting cell junction integrity (89). Its effect was studied *in vivo* in transgenic HLA HCD4/DQ8 mice. These are knock- out mice which with pre-sensitization to gluten (90). Larazotide countered the intestinal barrier disintegration, decreased the macrophage count in the lamina propria, and kept the transmembrane conductance intact, in mice that were given larazotide compared to the mice who were challenged with gliadin without larazotide (91).

Phase 1 trials (NCT00362856) of Larazotide acetate were conducted on 24 CeD patients that were challenged with 2.5 g of gluten after being on a GFD for at least 6 months. The results showed that 12 mg doses of larazotide decreased the cytokine response and intestinal permeability, as seen using the urinary LAMA (lactulose to mannitol) extraction fraction, although this difference was not statistically significant. The urinary LAMA ratio is an experimental biomarker used to quantify changes in intestinal permeability in research settings. In CeD, increased permeability due to mucosal injury leads to a reduction in absorption of monosaccharides (e.g., mannitol) and an increase in the paracellular absorption of disaccharides (e.g., lactulose). This results in an increase in the ratio of lactulose to mannitol excreted in urine after oral consumption of an aqueous solution of lactulose and mannitol (92).

Phase 2a study on 86 patients assessed the effect of larazotide on urinary LAMA ratio, the gastrointestinal symptom rating scale, psychological General Well-Being Index as well as adverse event profiling. It was a double blind RCT, testing 4 doses of larazotide acetate- 0.25, 1, 4, and 8 mg in patients who had been adherent to GFD and negative to EMA and anti-tTG Ab at the baseline. There was no significant effect of larazotide on the primary efficacy endpoint after gluten challenge which was the LAMA ratio. There was a definite decrease in the gastrointestinal symptom rating scale at two doses of 0.25 and 4 mg, but not in the other dose groups of 1 and 8 mg. Larazotide was generally well-tolerated by patients with the significant adverse events being headache and urinary tract infections in more than 5% of the patients; however there were no dropouts on account of these events (93).

In Phase 2b trials, 1, 4, and 8 mg doses of enteric- coated multiparticulate beads of larazotide were given to patients in a placebo controlled study. Results showed no statistically significant difference in the LAMA levels. The 1 mg but not the 4 or 8 mg doses of drugs brought a reduction to gastrointestinal symptoms in response to gluten (94). In a later phase 2 trial which tested 0.5, 1, and 2 mg doses of larazotide (NCT01396213), primary end point of reduction of symptoms was met in the 0.25 mg dose but not in the higher doses. The pattern that has therefore emerged in all the above studies is that only the lower doses had an effect, implying an inverse-dose relationship. Peptide aggregation at higher doses of the drug, reducing activity *in vivo* may be one of the possible explanations for this observation (95). Therefore, in the near future, this drug is expected to be tested in phase 3 trials on 924 patients who have been on GFD, using enteric coated, lower doses of 0.25 mg TID and 0.5 mg TID for 16 weeks (NCT03569007).

Approach 4: Tissue Transglutaminase- 2 Inhibition in the Intestinal Mucosa

Tissue Transglutaminase Inhibitors

Inhibitors of human tissue transglutaminase 2 (tTG-2) have been designed to prevent the conversion of gliadin to deamidated gliadin peptides, since the latter possess enhanced immunogenicity as antigens in CeD. tTG-2 is a multi-functional enzyme that catalyzes the linkage of glutamine and lysine side-chains to modify proteins, in the presence of ionic calcium and thiol. The enzyme is known to be associated with pathogenesis of not only CeD, but also some cancers (96) and, Parkinson's (97), Alzheimer's (98), and Huntington's diseases (99); hence tTG-2 inhibitors are being tested in many of the aforementioned conditions.

Three broad varieties of tTG-2 inhibitors have been well-described so far, namely competitive amine inhibitors, reversible inhibitors, and irreversible inhibitors. Competitive inhibitors compete with other natural amine substrates for tTG-2, thereby making the active enzyme unavailable for transamidating gliadin. These include putrescine, cystamine, spermidine, histamine, and cadaverine analogs like monodansylcadaverine. Currently the only commercially available tTG-2 inhibitor, mercaptamine or cystamine, has not been explored for its potential role in CeD (100, 101).

Reversible inhibitors inhibit substrate access to the active site without covalently modifying the tTG-2 enzyme. Examples in this class include, GTP, GDP, and their analogs, GTP-γS, βγ- methyl GTP, ionic zinc, as well as the recently discovered drugs with thieno [2,3-d]pyrimidin-4-one acyl hydrazide backbone (102).

The third group viz. irreversible inhibitors covalently bind to the cysteine in the active site and block the transglutaminase enzyme. Examples include iodoacetamide (103), -diazo-5-oxo-norleucine (DON) (104) and 3-halo-4,5-dihydroisoxazoles (105). The latter two compounds are based on the structure of acivicin, a natural analog of gliadin. Among all the above compounds, the dihydroisoxazoles and DON are selective for tTG-2 inhibition, with Ac-PQP-(DON)-LPF-NH being the most potent and selective. Also, it has been found that the 5-(S)-dihydroisoxazole is a markedly better inhibitor of human tTG-2 than its 5-(R) stereoisomer *in vitro* (106). A prototype dihydroisoxazole called 1 b, showed good oral bioavailability, efficient tTG-2 inhibition in small intestinal tissue, and low toxicity in animal studies (105). KCC009 is a dihydroisoxazole that has been studied for its application in cancers (107, 108). Other examples of dihydroisoxazole are ERW1041E (109) and R294 (110).

Also, several gluten- mimetic peptides have been developed with DON. They have a gliadin peptide sequence, where glutamines are substituted with the DON warhead (111). Gluten peptide analog ZDON (Zedira pharmaceuticals) has been shown to have a very high specificity for tTG-2. The inhibitors developed using ZDON, viz. ZED1098, ZED1219, and ZED1227 (Zedira pharmaceuticals) have demonstrated good solubility and stability in gastrointestinal conditions as well. These three compounds covalently bind to active site cysteine of tTG-2 (112). Among

these, the ZED1227 has been studied in phase 1 trials. Reports suggest that it is safe and also decreases the activity of tTG-2 and inflammation of bowel mucosa in mice models (113). Recently plans for phase 2 a trial (EUDRA CT No. 2017-002241-30) were announced by Zedira pharmaceuticals.

The guiding principle in the applicability of the above inhibitors is that they should attain in the lamina propria, a sufficient concentration without inhibition of other members of the transglutaminase family. Theoretically, the inhibition of tTG-2 can result in cross inhibition of other members of the transglutaminase family, several of which are indispensable for pathways like the coagulation cascade and the maintenance of epidermal integrity. Some studies suggest that transgenic mice with tTG knockout develop autoimmune diseases like glomerulonephritis (112). Since some gluten peptides are potentially immunogenic even without deamidation by tTG, combining tTG inhibitors with other pharmaceutical agents that eliminate immunogenic peptides before they enter lamina propria would be logical.

Si RNA Based Therapy

Si- RNA contained in gelatin based nanoparticles have been tested *in vitro* on intestinal epithelial (Caco-2 or the continuous cell line of human colorectal adenocarcinoma) cells. These silencing RNA molecules target human tTG-2 and IL-15, both of which are incriminated in CeD pathogenesis. In a study, fluorescent microscopy revealed that these spherical particles are rapidly internalized within 2 h and localize themselves to the cytoplasm. A 60% reduction in the gene products of tTG-2 and IL-15 genes was noticed 72 h after administration, which in turn translated into reduction in IFN-γ and TNF-α levels (114). Si-RNA appears to be a promising tool, although the effects of IL-15 suppression and suppression of other glutaminases may be a cause of concern. Regardless, this approach may be emulated to block other specific targets in the CeD pathogenesis in the future as well.

Approach 5: Prevention of Downstream Immune Activation After Gluten Exposure

HLA Blockers

HLA blockers prevent the interaction of antigen presenting cells (APC) that process and present gluten immunogenic epitopes using their MHC-II ligands which are a part of the HLA system, to the T Cell Receptor (TCR) of CD4+T helper cells. This amounts to prevention of an immunotoxic cascade that is a part of adaptive immunity in CeD. Cyclic peptides have been designed for blocking the gliadin binding groove of HLA-DQ2. These peptides are structurally similar to gliadin and compete with it to bind HLA-DQ2. These have the gliadin like epitopes-LQPFPQPELPY, KQPFPEKELPY, or LQLQPFPQPEKPYPQPEKPY and are cyclized using sulfide or polyethylene glycol bridges. Cyclization of these peptides results in effective blockade of the HLA grooves. Similarly, dimeric peptides with gliadin scaffolds have also demonstrated effective blockade. However, it has not translated into reduced T cell activation (115). Furthermore, ubiquitous requirement of HLA in various immune responses and a potential interference by the peptides in that function is one of the main concerns of use of HLA blockers.

Vaccines and Tolerogenic Therapies

The gluten products, upon entering lamina propria are recognized as immunogenic, and this triggers an adaptive immune response. Tolerogenic therapies using vaccines have been developed to hypo-sensitize the adaptive immunity.

Vaccine therapy

Hyposensitization is a well-known therapy for allergic diseases and is a potential therapeutic approach in autoimmune diseases as well. In a departure from their traditional use of immunization, vaccines are now being tested for desensitization. Examples of the latter include dendritic cell therapy in multiple sclerosis and in the case of Ced, the peptide-based vaccine called NexVax2. It was developed by a US based company, ImmunoSanT, Inc. NexVax2 is composed of three proprietary, immunodominant gliadin peptides named NPL001, NPL002, and NPL003 each of which is 15–16 amino acid long. The vaccine target is the HLA-DQ2.5-epitope-TCR complex linking the antigen presenting cell to the gluten-reactive CD4+ T cells.

It engages specific immune cells and a signature pathway has been discovered based on that. In animal studies in HLA-DQ2.5 transgenic mice having gluten-sensitive T cells, it was found to be efficacious (116). In a phase 1a study, 40 well-controlled CeD patients were given weekly intradermal injections of increasing doses of up to 90 μg of NexVax2 for 3 weeks. While there were some gluten-related gastrointestinal side effects, dose escalation could be completed, and safety was acceptable (117). Similarly in phase 1 b study, gradual dose escalation of up to 900 μg was tolerated well (118). Vaccinated subjects showed the target T cells becoming functionally unresponsive to antigenic stimulation with gluten challenge, consistent with immune tolerance. Nexvax2 was also associated with trends toward improved duodenal mucosal histology. Plasma concentrations of Nexvax2 peptides were dose-dependent (119).

Currently a phase 2, quadruple blind RCT (NCT03644069) is underway that will test 32 doses of twice weekly subcutaneous administration of this vaccine in subjects over a 16 week period. It will test the efficacy and improvement in CeD patient reported outcome score, as well the safety, with a follow up of 4 weeks post administration of the last dose.

The obvious limitation of vaccines is that they can engage only known or previously investigated immunogenic epitopes. Other aspects, such as efficacy and long-term safety, are to be established before peptide vaccines are made available for the management of CeD. Also, the effect profile in pediatric age group remains to be established. However, if successful, it has the potential to have prolonged benefits on patients.

Oral gliadin based tolerogenesis

Another way of inducing immune tolerogenesis, is the use of oral agents that act locally in the gut. *Lactobacillus lactis* has been engineered to release modified, non-toxic gliadin. A genetically engineered form of this non-colonizing, non-pathogenic bacterium was orally administered to secrete a

deamidated DQ8 gliadin epitope in the intestinal lumen of transgenic NOD-2 mice with ABoDQ8 haplotype. This induced suppression of the lamina propria and systemic DQ8-restricted T-cell responses, downregulation of IL-12 secretion, systemic production of IL-10 and TGF-β and induction of Foxp3+ Tregs in the lamina propria. These findings suggest development of mucosal tolerance to the gliadin (120).

Similarly a study used *Bacillus subtilis* spores as a long-lived, protease-resistant adjuvant system for administering gliadin peptides to HLA-DQ8-transgenic mice. The spore-adsorbed gliadin activated the dendritic cells and elicited a T-cell response in the gut. This mechanism (121) can be utilized for developing immune tolerance.

Tolerogenic immunomodulatory peptides.

Tolerogenic immunomodulatory peptides (TIMPs) are nano particles that may be used to deliver peptide epitopes intravenously to induce systemic immune tolerance. Gliadin containing TIMPs has shown favorable results in a single animal study (122). A drug called TIMP-GLIA (Cours pharmaceuticals) has been developed along these lines, having acquired a fast track approval from the FDA for phase 1 testing. The results of phase 1 trial are yet to be published, and the phase 2 trial is currently underway.

Cathepsin Inhibitor

Cathepsin is an intracellular protease that mediates apoptosis. A type of cathepsin called cathepsin S is expressed specifically in antigen presenting cells (APCs) where it mediates the proteolysis of the invariant chain. Invariant chain is an intracellular protein that prevents intracytoplasmic, self- antigen loading during the early stages of development of the MHC-II molecules of APCs. Once the MHC-II complex matures intracellularly, cathepsin S cleaves the invariant chain to permit antigen loading. Hence cathepsin S is important for the MHC II to function normally to present processed antigens to CD4+ cells (123). This mechanism is central to autoimmunity.

The cathepsin inhibitor, RO5459072 (also called RG7625), has been developed to target this pathway and is being tested in CeD, Sjogren's syndrome and other autoimmune diseases. A study (NCT022953320) showed that this drug resulted in a decrease in maturation of MHC-II bearing B cells and dendritic cells (124). The effects of RO5459072 on the immune response to gluten challenge in CeD patients has been investigated in a phase 1, placebo-controlled RCT (NCT02679014). Volunteers with previously diagnosed CeD were randomized to receive either 100 mg of RO5459072 or placebo twice daily for 28 days. The results of the study are awaited. Another cathepsin inhibitor in the pipeline is RG7236.

Immunosuppressants

CeD is similar to inflammatory bowel disease in being a chronic inflammatory condition with a similar profile of inflammatory chemical mediators. A major difference is that in CeD, abrogation of immune cascade occurs with GFD, IBD on the other hand requires lifelong immunosuppression. However, a subset of CeD patients require immunosuppression as an adjunct to GFD to enhance their recovery; these are patients suffering from refractory CeD, celiac crisis, and gliadin shock. The following are some of the anti-inflammatory and immune modulating drugs are being investigated in the context of CeD.

Glucocorticoids

Early historic reports on the inhibitory effect of steroids on B and T cell proliferation and on release of lymphokines by cultured cells based on *in vitro* studies, indicated that steroids may be effective in CeD (125). Wall et al. then showed an accelerated improvement in symptoms by addition of prednisone to GFD in patients with CeD (126). In another *in vitro* study, Mitchison et al. had also demonstrated the preventive effects of fluticasone propionate on the immunotoxicity of gluten (127).

Among the later studies, a proof of concept RCT evaluated the effects of a short course of prednisolone along with GFD on the markers of apoptosis and anti-apoptosis pathways, as well as on epithelial cell regeneration in treatment naïve CeD patients. The hypothesis was that addition of prednisolone for a short term, would accentuate the mucosal recovery. A 4 week course of 1 mg/kg/d prednisolone in addition to GFD was given to treatment naïve CeD patients. It was found that compared to baseline, patients on both GFD alone control group as well as the GFD plus prednisolone group had underexpression of the markers of apoptosis viz. p53 and M30. However, all apoptotic markers other than H2AX showed a rebound increase 4 weeks after cessation of steroids, indicating that steroids may have indeed played a role in suppressing apoptosis and epithelial injury, during the period where the steroid was available in the systemic circulation. The study also found that steroids could however counterproductively lead to a suppression of proliferation index and therefore of epithelial regeneration (128).

More recently, budesonide has piqued the interest of researchers in view of its potential application as a steroid with low systemic bioavailability and hence higher effect on the gut. Ciacci et al. studied the effect of oral administration of 6 mg budesonide capsules for 4 weeks on CeD patients on GFD. It was found that the drug significantly decreased stool frequency and stool weight and improved general well-being as indicated by subjects on a Visual Analog Scale, in comparison to those on GFD alone. The same study also investigated the effect of budesonide *in vitro* on intestinal epithelial cells from biopsies of CeD patients, exposed to gliadin and its toxic fraction p21-43. Budesonide significantly decreased the expression of inflammatory markers ICAM-1, COX-2, and HLA-DR (129).

Steroids in refractory CeD

Brar et al. studied retrospectively, 30 CeD patients who were prescribed budesonide while following up for symptoms that were refractory despite maintaining adherence to GFD. They noticed, that 75% of patients had at least partial response to budesonide ± either azathioprine or systemic steroids, and 55% responded completely. Complete response was higher among those with secondary refractory CeD i.e., in those whose disease manifestations recurred after an initial response to a GFD, compared to those whose disease remained refractory from the time of institution of the GFD (primary refractory CeD) (130).

In another recent series, Mukewar et al. showed clinical (92%) and histologic (89%) improvement in 57 patients with refractory CeD who were treated with oral budesonide. Half of these patients had earlier been treated with immunosuppressive therapy with no or incomplete response. Follow-up biopsy in 7 out of 13 patients with refractory CeD-2 (RCD-2) (53%) showed an absence of clonal TCR gamma gene rearrangement/aberrant IEL phenotype that was previously seen (131). Similar response was also reported by Daum in patients with refractory CeD (132).

Stem cell therapy

Intestinal epithelial cells in the crypts and villi are in a state of continuous turnover with stem cells from the base of crypts proliferating, differentiating into mature phenotypes and rising to the outer villous surface to replenish the older cells that are shed. These stem cells are identified by the markers CD133+/Lgr5+. There have been case reports of using hematopoietic autologous stem cell transplant being successfully used to treat the enteropathy of CeD patients (133). Autologous stem cell transplant has been tried in patients with refractory CeD as well. In a pilot study, 13 patients with refractory CeD were subjected to ASCT, it was found that not only was ASCT well tolerated, but also it led to rapid clinical response which lasted for at least 2 years in patients (134). Furthermore, in a series amongst 54 patients who had been diagnosed with enteropathy associated T-cell lymphoma from 1994 to 1998, 14 underwent ASCT. All of them showed disappearance of intestinal lesions as well as prolonged remission of the disease (135). However, occurrence of neutropenia and other complications of stem cell transplant are likely to affect the acceptability of this therapy except in some refractory cases.

CCR9 antagonists

In order for immunoreactive T cells to home in to the intestinal mucosa and cause celiac autoimmunity, they use their ligand called CCR9 to bind with the receptor CCL25 on the intestinal mucosal epithelium. Antagonists to CCR9 have been developed, e.g., CCX8037 and GSK-1605786 (CCX-282; Traficet-EN, Vercirnon). The latter is a drug that is being developed for potential use in Crohn's disease and CeD. This molecule was characterized and tested *in vitro* by Walters et al. on Molt-4-T cell lines which endogenously express CCR9. Stimulation of Molt-4 cells with CCL25 resulted in release of intracellular ionic calcium which in turn promoted chemotaxis. Also, CCL25 is a chemoreceptor specific to intestinal mucosa. The antagonist to CCL25 prevented the interaction of chemokines CCL25 and CCR9, and prevented release of calcium and homing- in of inflammatory T cells. The same study also found the results to be replicable in mouse models where intestinal inflammation was attenuated in response to the drug. Furthermore, the drug CCX282 was found to be highly selective for CCL25-CCR9 interaction (136). Tubo et al. have also tested this drug in mouse models and found similar results. Interestingly they also inoculated this drug into inflamed ear skin in mice, and found no attenuation of inflammatory response in the draining lymph nodes showing that this drug would specifically act in the immune system of the intestine and spare other immune organs (137).

A RCT (NCT00540657) to study the efficacy of administration of 250 mg of oral CCX282 twice daily in comparison to placebo has been completed in patients with CeD on 24 months of GFD. The outcome measures included effects on V:C ratio and markers of intestinal inflammation, serology and symptoms upon gluten exposure. The results of this study are yet to be published. While a phase 2 clinical trial of CCX282 in patients with Crohn's disease (NCT00306215) had given encouraging results (138), the phase 3 trials failed to show any significant difference of results compared to placebo (139).

Interleukin-15 antagonists.

IL-15 is overexpressed in the intestinal mucosa in CeD on exposure to gluten. It mediates the inflammatory response that leads to intestinal epithelial damage. Humanized Mik-Beta-1 monoclonal antibody has been developed to target IL-2/IL-15R Beta (CD122). Hu-Mik- Beta-1 is currently being investigated for safety in phase 1 trials in a three dose regimen (Day 1, week 3, week 6) in refractory CeD patients.

Another human monoclonal antibody against IL-15, with a similar mechanism of action is AMG 714 (140). This drug has been developed by the biotechnology company, Amgen. It is being investigated in phase 2, double blind placebo controlled, dose- varying RCT.

Integrin antagonists.

Vedolizumab is a humanized monoclonal antibody that prevents the interaction of anti-α4β7 integrin with mucosal addressin cell adhesion molecule-1 (MAdCAM-1). The latter is found exclusively on the intestinal mucosa, enabling vedolizumab to prevent chemotaxis of memory T cells into the mucosa from the circulation. This specific chemotaxis directed toward mucosa or homing- in of T cells is responsible for the chronic inflammation seen in IBD. Vedolizumab is therefore used for the management of patients with Crohn's disease and ulcerative colitis. Similar to IBD, there is an overexpression of α4β7 integrin in patients with CeD and hence its effect in CeD is worth exploring. Vedolizumab is under a phase 2 clinical trial (NCT02929316) in CeD patients, with results expected in 2019.

Immunomodulator drugs with potential application in CeD

Other potential immunomodulators include anti- TNF-α agents like infliximab. Infliximab has been shown to be useful in case reports of refractory CeD (141, 142), however a larger human trial is yet to be done. Similarly natalizumab has also been tried in patients with multiple sclerosis or IBD in addition to CeD (143), with induction of remission in both CeD and the co-occurring condition.

Mulder et al. studied the use of recombinant IL-10 for treating patients with refractory CeD and found that only 2 of the 10 treated patients responded histologically (144). Another potential immunomodulator is the anti-CD20 antibody, rituximab. While it is worth exploring the use of this agent in CeD to abrogate the abnormal mucosal plasmablasts and antibody formation, a

study found no significant effect of rituximab on mucosal B cells (145).

Other Therapies Under Investigation

Besides the five approaches discussed in the preceding sections, there are a few experimental therapies that modulate factors known to be associated with CeD but as yet are not known to play an obvious role in its pathogenesis. These therapies include probiotics and prebiotics, the low FODMAP diet, pancreatic enzyme supplements, and helminth therapy using hookworms. Probiotics have been tried in patients with CeD, since studies have found a reduction in the relative proportion of Firmicutes, Proteobacteria, Bifidobacterium and a relative increase in Bacteroides and E.Coli in CeD patients compared to controls. A few RCTs have found that probiotics lead to improvement in symptoms in CeD (146) while some animal studies have found *Bifidobacterium* and *Lactobacillus* to reduce gluten-induced toxicity. The proprietary probiotic formulation VSL#3 has demonstrated activity in gliadin pre-digestion in a study (147). Similarly, a study found that oligofructose-enriched inulin (Synergy 1), a prebiotic, increased the Bifidobacterium count in the gut significantly, with no side effects (148). These findings point to a possible causative role of gut dysbiosis in CeD, although the exact mechanism remains obscure. Studies are also exploring the use of low FODMAP (Fermentable oligo-, di-, mono- saccharides, and polyols) diet, which is low in short chain polysaccharides like fructans, lactose, mannitol, sorbitol etc. These sugars are hard to digest, resulting in fermentation in the bowel and flatulence, and are implicated in causing some of the symptoms of Irritable Bowel Syndrome (IBS) (149). The low FODMAP diet may therefore be beneficial in CeD, especially in those with functional IBS like (150–152) symptoms. One limitation of this diet is that it is even more restrictive than GFD and can increase susceptibility to nutritional deficiencies. It is also noteworthy that much of the evidence concerning the use of low FODMAP diet is derived from moderate to low quality studies in IBS patients where proper blinding and controls have not been used, with uncertainty over whether the benefit of the diet is a placebo effect (153). In another experimental approach, Weiss tested the hygiene hypothesis of autoimmunity by investigating the effect of hookworm *(Necator americanus)* therapy on CeD. The hygiene hypothesis has been derived from the observation that there has been a simultaneous decrease in infectious diseases and an increase in autoimmune diseases globally (154). Chronic low grade helminthiasis, while inducing specific immune response to itself, also diminishes Th1 cells response to other antigens (155), which can in turn suppress autoimmunity. Daveson found that inoculation of hookworms into the skin increased microbial richness in the fecal samples in the same patients. It is possible therefore that helminthic infection modulates CeD pathogenesis through an unknown mechanism that could be similar to probiotics (156–159). Furthermore, they found hookworm therapy to be safe and well-tolerated with none of the patients developing significant anemia. Administration of pancreatic enzyme supplements may also be helpful in a subset of CeD patients classified as having Non Responsive Celiac Disease or NRCD (persisting symptoms of CeD despite 6–12 months of adhering to a GFD). This is because exocrine pancreatic insufficiency may be one of the factors known to contribute to NRCD symptoms, other factors being microscopic colitis, refractory CeD, dietary lactose/fructose malabsorption and small intestinal bacterial overgrowth (147). A RCT is currently underway to test the effect of pancreatic enzyme supplementation in NRCD patients (NCT02475369).

CONCLUSIONS

GFD is likely to remain the mainstay of therapy of CeD in the near future, since all other treatment modalities are only in the preliminary stages of research. An ideal therapeutic agent would be one that permits a CeD patient to consume gluten in usual amounts, without compromising her/his quality of life. So far, only vaccines have come closest to the potential of helping patients achieve that ideal state. Vaccines also show promise in terms of bearing prolonged benefits, subverting the need for gluten restriction all together. Glutenases are another group of drugs that have been extensively explored as therapeutic agents and their list is growing with newer discoveries. Among these, latiglutenase had reached the farthest in terms of clinical trials; although the most recent clinical trial on a large sample size delivered disappointing results. Results of preliminary studies on another glutenase, Kuma030, suggest that this enzyme may hold more promise in future, compared to other glutenases studied so far. Similarly, large scale testing of the zonulin antagonist, larazotide acetate with lower doses could yield new insights into its effectiveness, due to results of previous smaller studies demonstrating an inverse dose- response relationship. To conclude, although most trials on novel therapeutics are currently in phase 2 or earlier stages, ongoing research in areas targeting various molecular pathways in CeD is robust. This leaves much scope to find definitive alternatives to GFD in the years to come, in order to improve the quality of life of patients with CeD.

AUTHOR CONTRIBUTIONS

SY: review of the literature, drafting of the manuscript, conceptualization of illustrations. GM: concept building, critical review and finalization of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00193/full#supplementary-material

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REVIEW
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Changing Pattern of Childhood Celiac Disease Epidemiology: Contributing Factors

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Up until the 1960s and 1970s, diarrhea, malabsorption syndrome, and failure to thrive were the presenting symptoms and signs of celiac disease (CD) in young infants; however this disease was also at the same time reported to be disappearing. Indeed, clinical childhood CD was seen to transform into a milder form, resulting in an upward shift in age at diagnosis during the 1970s (and years later for many countries). This changing pattern of CD presentation then altered the epidemiology of the disease, with major differences between and within countries observed. An awareness of the changing clinical nature of CD and use of case-finding tools to detect even clinically silent CD became an important factor in this changing epidemiology. Countries report both low and high prevalence but it seems to be on the increase resulting in a population-based level of 1–2%. This paper discusses the potential causes and environmental factors behind these observed clinical changes, identifying new clues from different studies published at the time this transformation took place. For instance, it was found that breastfeeding postponed the diagnosis of the disease but did not altogether prevent it. Moreover, gluten introduction at a young age, specifically at the mean age of 2 months, seemed to also have a clear impact in inducing malabsorption syndrome and failure to thrive in young infants in addition to other factors such as gluten intake volume and type of cereal present in the weaning food. Further, the impact of cow's milk and its high osmolarity might have played an important role; humanized milk formulas were not yet invented. Future epidemiological studies on the contributing environmental factors to the shift in CD presentation are thus recommended for countries in which these changing clinical features are still being observed.

Keywords: celiac disease, changing pattern, incidence, prevalence, infant feeding, autoimmunity, awareness, screening

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INTRODUCTION

Celiac disease (CD) has earlier been considered to be a rare intestinal disease occurring only in children, a disease that Samuel Gee in 1888 presented as the "coeliac affection" (1). As summarized by John Walker-Smith, Gee was very accurate in his description of childhood CD: "it is a kind of chronic indigestion, which is especially apt to affect children between 1 and 5 years old and where signs of the disease are yielded by the feces, being loose, but not watery, bulky, and pale.

The onset of the disease is usually gradual and cachexia is a constant symptom. The belly is mostly soft and often distended" (2). The conception on CD changed in early 1950s by the discoveries of Dicke, who showed that the cause of harm in patients with CD was dietary gluten; in fact, he specifically noted that the disease was not caused by all cereals but, specifically, wheat flour (3). The next major research discovery occurred in the 1950s and involved the use of a peroral intestinal suction biopsy apparatus as a diagnostic tool. When gluten was ingested by children with CD, the development of a characteristic mucosal lesion in the jejunum was observed, namely villous atrophy with crypt hyperplasia (4). Moreover, new biopsy criteria were adopted early on in Europe by the European Society for Pediatric Gastroenterology, even for young infants (4–6).

Celiac disease (CD) was long thought to be a rare disease, occurring only in children with a classical presentation known as malabsorption syndrome and failure to thrive (7). However, today, CD is known as an autoimmune systemic disorder occurring in genetically susceptible individuals and perpetuated by the daily ingestion of gluten cereals (i.e., wheat, rye, and barley) with manifestations both in the small intestine and in extraintestinal organs (7–10). CD is more complex than simple intestinal malabsorption, which is, in fact, no longer essential for diagnosis. Another important milestone in CD diagnostics and screening was the discovery and clinical use of highly CD-pathognomonic, circulating, gluten-dependent tissue autoantibodies measured within the immunoglobulin A (IgA) class (11–18). Awareness of the disease and use of these tools have come to determine the epidemiological outcome in CD research. Furthermore, late-developing, small-intestinal mucosal injury also plays a role in the changing pattern and epidemiology of CD. In other words, this refers to a CD latency in which the disease exists but is not manifested at the mucosal level, even if the patient has ingested gluten for decades (9). Susceptibility to CD is inherited; however, upon a child's first encounter with gluten he may or may not develop the disease at a small-intestinal mucosal level soon thereafter. Oral tolerance toward gluten can persist for a long period of time with the deterioration of the mucosa possibly taking place at a later age. This also explains the late mucosal relapses during gluten challenges and late appearing mucosal injury concordance in monozygotic twins as well as possibly spontaneous mucosal recoveries upon continued gluten ingestion.

Celiac disease (CD), known as a rare malabsorption syndrome in young Caucasian children has, since the 1970s, come to be known as a common, chronic, food-induced autoimmune systemic disorder world-wide, diagnosed in children, adolescents, adults, and the elderly. The changes in perception of CD as a clinical disease and observed changes in occurrence have been remarkable during the past 50 years. In these aspects, there are, however, several differences between countries and continents. This review thus discusses the pattern of change in childhood CD presentation, its influence on its epidemiology, increasing incidence rates, and the causes behind them.

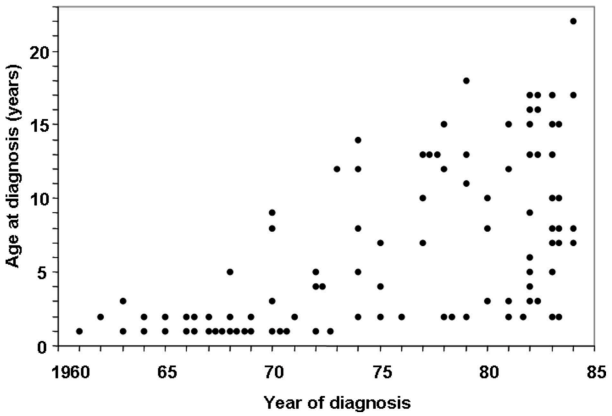

FIGURE 1 | Changing pattern of childhood celiac disease affecting epidemiology results in Finland in 1960–1984. Decreasing numbers of cases were diagnosed in infancy and increasing numbers diagnosed at school age and adolescence after 1972. [Reproduced with permission from Mäki et al. (23)].

CHANGING CLINICAL FEATURES

In the 1960s, a child with CD typically presented with prolonged diarrhea, malabsorption syndrome, and failure to thrive, often before the age of 2 (7). However, a shift in CD presentation became evident during the 1970s and 1980s, with childhood CD reportedly disappearing altogether (19–22). In Finland, however, from 1970 onward, it was observed that, in addition to the disappearance of the classical form of CD in young children, the incidence rates of the milder forms of the disease increased, resulting in an upward shift in age at diagnosis from 2 to 8 years (**Figure 1**) (23). This was also evident in the UK, with a changing disease pattern seeming to be a fact; childhood CD tended to present at later age (24).

When CD manifests during the later stages of childhood, symptoms can be gastrointestinal; however the disease is often monosymptomatic, presenting with short stature, delayed puberty, and joint pain or anemia due to isolated iron deficiency (23, 25–30). Gastrointestinal symptoms may also be mild or non-existent. If the healthcare professionals are diagnosing only classical CD presenting at young age and older children with gastrointestinal symptoms, only the tip of the iceberg of all CD patients are detected (**Figure 2**), epidemiologically resulting in low incidence and prevalence numbers. These clinically silent patients, i.e., children with no symptoms or signs suggesting CD, also exhibit a gluten-triggered and -dependent small intestinal mucosal lesion manifestation (**Figure 2**). They require an increased awareness of healthcare professionals and a case-finding strategy for early diagnosis. Specifically, celiac autoantibody testing can determine an underlying gluten-induced disease, yielding high sensitivity and specificity (11–18). The existence of clinically silent patients became evident when performing gluten challenges to earlier diagnosed well-treated patients (31, 32) in addition to CD screening for certain risk groups, such as those with type 1 diabetes (13, 18) or

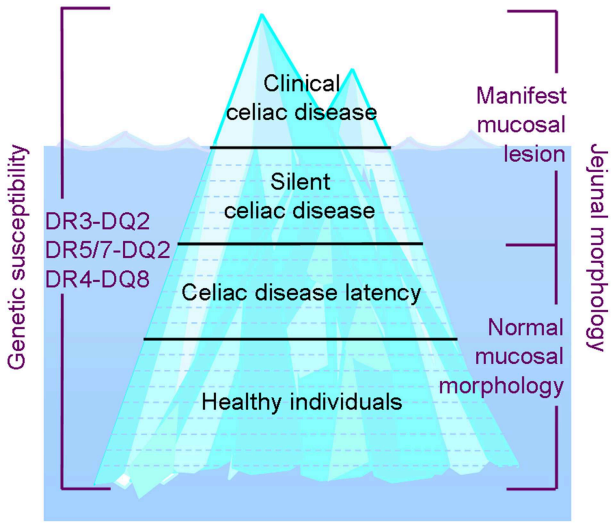

FIGURE 2 | The celiac disease (CD) iceberg and the spectrum of celiac trait: Awareness of different clinical forms of CD and clinicians' activity in case-finding by screening will influence the outcome of incidence rates and prevalence in different countries and within countries.

healthy, first-degree family members (33, 34). Case-finding using celiac-pathognomonic serum IgA class R1-type reticulin autoantibodies (11, 12), later known as endomysial and tissue transglutaminase antibodies (14, 15), detected the clinically silent patients and helped change the perception of childhood CD. The changing pattern of CD epidemiology would partly go unnoticed, if healthcare professionals are not aware of the existence of clinically silent CD and use a case-finding strategy in diagnostics.

Latent CD refers to the fact that the disease exists but does not manifest at the mucosal level until at some later age. The small intestine mucosa can stay intact upon years and decades of full gluten ingestion, or the mucosal damage can develop gradually. Final deterioration of the mucosa occurs when oral tolerance toward gluten ceases (9, 35) (**Figure 2**). We have extensively discussed latent and developing CD in a recent review article (9). Latent CD patients may be serum autoantibody positive, have a normal small intestine mucosa and thus according to biopsy criteria be excluded for CD. These patients may even become serum autoantibody negative upon normal gluten ingestion but still develop the disease at some later age (36). Stenhammar et al. addressed the latency issue in CD family studies spanning 20 years (37, 38). When a small intestine biopsy was performed for 100 first-degree relatives of index patients with childhood CD in 1982, only 2% were found to have clinically silent CD. When they reinvestigated all first-degree relatives of the same index patients 20–25 years later, eight new cases of CD were detected (8.3%), two of whom were found not to have the disease in the first study. Eleven more relatives exhibiting mild enteropathy were identified in the new screening, and it was suggested that they were followed carefully for potential development to overt disease. CD incidence and prevalence figures are influenced by the age of the patients at time of study and also whether a changing pattern of childhood CD has taken place in the country or setting where the study was performed.

A large European study involving 38 centers and 5,871 children with diagnosed CD confirmed that there had been an overall mean increase in age at diagnosis from 2 years in 1975 to 4 years in 1990 (39), with Sweden being the exception (40). The changing CD pattern has been described as occurring at different times within and without different countries. In the Naples region of Italy, for example, no change in age at diagnosis was observed between 1973 and 1986 (41). In some other parts of Italy, however, an increase in age was observed (39), with classical forms of CD decreasing in the late 1990s and early 2000s and silent forms significantly increasing, resulting in an increased age at diagnosis as well (42). Similarly, no changes in the overall clinical makeup of CD could be found in the Netherlands between 1975 and 1994 (43) but an increased awareness and recognition of childhood CD did ultimately change the clinical presentation during the years 1993–2000 (44). In the UK, considerable changes were reported mostly due to targeted serum antibody screening. A decrease in the proportion of patients presenting with gastrointestinal manifestations and a rise in the number of patients without symptoms was also observed (45). This change was still occurring in the UK during the years 2005–2011, with over 50% of children diagnosed with CD exhibiting few or no symptoms (46). This same type of changing clinical pattern has been observed in Greece (47), North America (48–50), India (51, 52), Estonia (53), Turkey (54), and Sweden (although, in this case, only after mid-1990s when the Swedish epidemic occurring in young infant from 1985 to 1995 had ended) (40, 55). In Finland, between 1975 and 1990, large differences in CD incidence rates were found when comparing Helsinki to Tampere (39), since an awareness of clinically silent CD was not acknowledged by primary health care professionals in the capital of Finland. Later, however, in Helsinki, the number of patients with CD was found to increase six-fold between 2000 and 2005, with mean age at diagnosis increasing to 7.2 years (56). When comparing the two neighboring countries of Finland and Sweden based on CD's clinical features from 1985 to 1989, the differences found were remarkable: in Sweden, classical clinical symptoms and a diagnosis before the age of two was most commonly observed, whereas, in Finland, symptoms varied much more greatly, and diagnosis was often made after the age of 8 (57). However, the presentation of CD in Finnish children seems to no longer be shifting, with a plateau having been reached in recent years (58).

These observed changes in clinical presentation had inevitable effects on the epidemiological outcomes of CD and were, in terms of timeframe and magnitude, not similar between and among the different countries. It is thus difficult to compare different CD incidences from different publications and countries. Overall, a change in the pattern of symptom presentation for childhood CD that is in line with early observations has been noted in the textbooks as well as in CD guidelines worldwide (7, 59–61).

In Finland, during the 1970s, there was a growing awareness of the aforementioned changing CD pattern, as shown in **Figure 1**, which influenced health care professionals' case-finding via screening. In fact, this awareness seemed to be the major

factor involved in the changing epidemiology and increasing incident rate observed in older children. However, it should be noticed that changing epidemiology is also a result of whether disease-specific autoantibody tools are used for case-finding in the primary healthcare field instead of only in secondary or tertiary referral centers. Today, children with CD are not often so symptomatic that they would need to be admitted to pediatric gastroenterology centers. Furthermore, case-finding using autoantibody screening and a high index of disease suspicion is in our experience a prominent diagnosis method in primary care. Finland also experienced a decrease in the yearly incidence rate during the economic crisis of the early 1990s, during which the city of Tampere health authority forbade primary care doctors to use reticulin or endomysial autoantibodies as case-finding tools, suggesting instead the cheaper but outdated crude gliadin serum antibody test. The authority intervention resulted within a 2-year timeframe in a three-time decrease of biopsy-verified diagnoses at the referral center.

The current major question in pediatrics asks what has caused the described change in the clinical presentation of CD over time. When addressing the changing epidemiology of childhood CD in 1992 (39), we suggested the following environmental factors to play a role: genetic background of the population, infantile gastroenteritis/other infections, unmodified/adapted cow's milk formulas, breastfeeding, age at gluten introduction, quantity of gluten, quality of cereals, and quality of wheat gluten (62). These environmental factors will be discussed here in more detail. The present review is not extended to include human leukocyte antigens (HLA), non-HLA genes, or other genetic aspects in populations with low prevalence rates of CD, such as those comprised of black Africans or Japanese individuals, but did include environmental factors in populations with similar HLA DQ2 and DQ8 background. Environmental factors have an evident impact on intestinal epithelial cells, either through direct interaction or through microbiota. This review does not address any potential epigenetic mechanisms, such as histone modifications, DNA methylation, or microbial RNA methylation, as indicators of the changing pattern in CD epidemiology over time.

ENVIONMENTAL FACTORS BEHIND CHANGING PATTERNS OF CELIAC DISEASE

Infant Feeding Patterns

Breastfeeding

As early as 1953, Andersen and di Sant'Agnese found that breastfeeding for a duration of more than 2 months delayed the onset of diarrhea in patients with CD (63). Moreover, two case-control studies performed in Italy indicated breastfeeding protected patients against CD (64, 65). When evaluating whether the duration of breastfeeding influenced age at diagnosis, a mean increase in breastfeeding from 2.5 months in children born from 1961 to 1965 to 3.9 months in children born from 1976 to 1980 was observed. Furthermore, a significant correlation was found between age at diagnosis and duration of breastfeeding in a study of 45 children diagnosed with CD before the age of 6 years, with breastfeeding found to delay the onset of symptoms (23). Thus, prolonged breastfeeding seemed to prevent the development of CD but did not protect patients against the disease, since it manifest at a later age. Overall, the median age at diagnosis was 8.8 years in Tampere, Finland during the early 1980s (39). In the neighboring country of Sweden, at the time of their CD epidemic in 1985–1995 (described below) and a national trend in short breastfeeding duration (40), the median age at childhood diagnosis was 1.3 years (57). **Table 1** shows the percentages of Finnish, Italian, and Swedish children breastfed at the ages of 6, 9,

TABLE 1 | Amounts of ingested gluten-containing cereal proteins (g per protein per kg body weight per day) and percentage of breastfeeding of healthy Finnish (Tampere), Swedish (Gothenburg) and Italian (Naples) infants at 6, 9, and 12 months of age in 1990 at the time of the Swedish epidemic [adapted from Ascher et al. (57) and Mäki et al. (62)].

	n	Wheat		Rye		Barley		Oats		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Breast-feeding %
6 months										
Tampere	73	0.06[a]	0.06	0.02	0.03	0.01	0.02	0.20	0.23	71
Naples	50	0.44[a]	0.24							10
9 months										
Tampere	60	0.14[a]	0.09	0.08	0.10	0.03[a]	0.04	0.25[a]	0.17	47
Gothenburg	20	0.48[a]	0.21	0.04	0.05	0.01[a]	0.01	0.16[a]	0.14	20
Naples	58	0.6[a]	0.23							(1 child)
12 months										
Tampere	47	0.20[a]	0.13	0.12	0.12	0.03	0.03	0.23	0.15	17
Gothenburg	13	0.42[a]	0.13	0.09	0.05	0.02	0.02	0.20	0.19	0
Naples	58	0.60[a]	0.25							0

[a] *Significant difference between countries.*
In Naples no rye, barley, or oats was consumed.

and 12 months during a time in which the diagnoses of children under two displayed values of 7, 40, and 74%, respectively (57, 62). At the same time, in the UK and Ireland, it was observed that breastfed babies presented with CD later than their bottle-fed counterparts (22, 24). In 2002, Ivarsson et al. revealed the protective effect of breastfeeding against CD (66).

As the research has suggested, breastfeeding may prevent the development of CD. Thus, Akobeng et al. conducted a systematic review and meta-analysis of certain observational studies published between 1966 and 2004 (67). The meta-analysis showed that the risk of CD was significantly reduced in infants who were breastfed at the time of gluten introduction (pooled odds ratio of 0.48, 95% CI 0.40–0.59) compared with infants who were not breastfed during this period. A systematic review from 2012 provided a similar picture, with the study concluding that certain studies indicate a protective effect of breastfeeding, while others do not. The meta-analysis reported a lower risk of CD in breastfed infants, further stating, however, that it was unclear whether those breastfed received permanent protection from CD (68).

In 2016, a position paper by the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) stated that breastfeeding has not been shown to reduce the risk of CD in children (69). Indeed, new evidence on breastfeeding not to be protective against CD has emerged from prospective randomized clinical trials (70–72). However, the evaluated studies were not often designed to directly address the effect of breastfeeding on CD. At the time of the changing pattern in CD presentation, prolonged breastfeeding in Finland was associated with a postponed diagnosis of CD but not with its prevention altogether (23). This effect was also observed in Ireland and the UK (22, 24) at the same time, with breastfed infants diagnosed with CD later than their bottle-fed counterparts despite their similar ages at gluten introduction (24). These early reports indicate that breastfeeding could still have contributed to the changing epidemiology of CD (19–24).

Age at Gluten Introduction

In our study of the changing pattern observed in childhood CD presentation (**Figure 1**), no correlation between age at diagnosis and time of gluten introduction was found (23). Meijer et al. presented recent studies as evidence of the effect of gluten introduction timing and the associated risk of CD in young children (73), concluding that it did not influence the risk of CD, as also suggested by the results of large prospective studies. Furthermore, the position paper by ESPGHAN in 2016 recommended (a) to not introduce gluten while the infant is being breastfed as a means of reducing the risk of developing CD (i.e., decreasing the incidence rate of CD); (b) gluten introduction at age 4–6 months, compared to gluten introduction at age >6 months, does not reduce the cumulative incidence rate of CD in children; (c) for children at a high risk of CD, gluten introduction at 6 months of age compared to 12 months of age does not reduce the cumulative incidence rate of CD but instead leads to earlier manifestations; (d) it remains unclear whether gluten introduction at age <3–4 months compared with 4–6 months has an effect on the risk of developing CD; and (e) it remains unclear whether gluten introduction at age <3–4 months compared to gluten introduction at age >6 months has an effect on the risk of developing CD (69).

Even if the current available evidence suggests that age at gluten introduction is not associated with the changing CD epidemiology, this review suggests that it could still have played an important role at the time when chronic diarrhea and failure to thrive disappeared as presenting clinical features of the disease (19–22, 24). It should be noted that in the 1960s and earlier, gluten was introduced to infants at a very early age. From 1960 to 1965 in Ireland, the mean age at gluten introduction for 130 newly diagnosed children was 2.3 months, while, from 1976 to 1981, it was 4.2 months (22). Moreover, in the UK before 1975, the median age at gluten introduction for diagnosed CD patients was 2 months, while, after 1975, it increased to 4 months (24). These changes in infant feeding patterns coincided with the observed change in CD epidemiology.

Amount of Gluten and Quality of Cereals

The amount and type of cereals an infant ingests may play an important role in the changing epidemiologic makeup of CD. Since major differences in the incidence rate and presentation of CD in Finland and Sweden were observed, a joint study was conducted to explore potential causes of the phenomena (57). The results from late 1980s showed that the Gothenburg region of Sweden reported 30 times more infants diagnosed with CD before the age of two than Tampere, Finland. At this time in Finland, cereals were implemented gradually into infant diets from the age of 5 months onward, with parents having a choice between different types of gluten-containing cereals (i.e., wheat, rye, barley, oat) or non-gluten-containing cereals (i.e., rice) in the form of gruels or porridge. On the contrary, in Sweden since 1982, there has been a rapid introduction of gluten at the age of 6 months. **Table 1** shows the results of a prospective study of dietary intake of gluten-containing cereals in two neighboring countries exhibiting highly significant differences in the amount of gluten ingested and in quality of cereals (57, 62). The Swedish infants at the age of 9 months ingested three times and at the age of 1 year twice the amount of wheat protein compared to their Finnish counterparts (**Table 1**). The same evaluation was at the same time conducted in Italy in the Naples region. In Italy, weaning food was completely wheat-based. When wheat, rye, barley, and oat proteins are counted at once, the figures of ingested gluten of the Nordic countries converged with those of Italy. The gluten amount ingested during infancy also correspond to the country differences in CD incidence and prevalence at that time (23, 40, 41, 57).

Here it should be noted that "gluten" (i.e., avenin) in oats is not CD-inducing (74–76). Therefore, another important aspect to consider why in the late 1980s Sweden experienced 30 times more infants diagnosed with CD before the age of 2 than Finland, is the amount of oats ingested by the infants: at the age of 9 months, Finnish infants ingested significantly more oat protein than Swedish infants (**Table 1**).

Low wheat intake has been thought to cause the low incidence rate of CD in the countries of Denmark (77) and Estonia (78). When comparing the prevalence of CD in Denmark vs. Sweden

in 1987, it was estimated that, for infants at 8 months of age, the Swedish diet contained 40 times more gliadin than the Danish diet, while, at 12 months of age, it contained four times more. The weaning food in Denmark was based on rye not wheat (77). Rye gluten, i.e., secalin could actually be clearly less disease-inducing than wheat gluten, i.e., gliadin.

More recently, Cresco-Escobar et al. reported that gluten consumption patterns and the amount of gluten consumed between the ages of 11–36 do not influence CD development in children at a genetic risk for the disease (79). Furthermore, a new study from the US was able to link gluten intake volume in 1-year-olds with the future onset of CD (80).

One further potential environmental factor in influencing the presentation of CD might be the changes in the gluten-rich cereal varieties themselves because of wheat breeding. New hybridization techniques have been used to produce new strains of modern wheat where celiac-triggering gluten proteins are expressed to higher levels. These new strain do express even new gluten proteins on which no animal or human safety testing was conducted (81, 82).

Cow's Milk as a Contributing Factor to CD

The present review regarded also older publications from the time when changing clinical features were observed and infants were fed unmodified cow's milk. Important modern epidemiology literature using systemic reviews and meta-analyses has not discussed these studies. The third important contributing factor to changing CD epidemiology in infancy, in addition to the introduction of breast milk and gluten, might in fact be the use of cow's milk.

During the 1960s, young children from the Tampere region diagnosed with CD all received homemade, diluted, cooked, "half"- and "two-third"-cow's milk (23, 62, 83). The shift from using diluted cooked cow's milk to humanized, i.e., more breast milk resembling cow's milk-based infant formulas took place in Finland during the year 1972. This, together with the decrease in the amount of ingested gluten as a result of prolonged breastfeeding, could have significantly contributed to the disappearance of the symptomatic forms of CD in young children. In the UK, bottle-fed infants presented earlier with CD when compared to breast-fed infants (24). This was true both prior to 1975 when gluten was introduced at a median age of 2 months as well as after 1975 when gluten was introduced at a median of 4 months. Before the mid 1970s, most artificially-fed Irish infants received formulas containing unmodified cow's milk, with the total protein content and osmolarity of such formulas both reduced at the same time as the decreasing incidence rate of CD was observed. Humanized infant formulas were implemented from 1978 onwards both in the UK and Ireland (22).

Kuitunen et al. reported that malabsorption syndrome caused by cow's milk protein intolerance in patients from 1962 to 1971 was a clear-cut clinical entity; gluten was not the causing factor of the disease (84). Moreover, small-intestinal biopsies obtained from these infants showed that the jejunal mucosa was injured, the patients displayed villous atrophy with crypt hyperplasia that was undistinguishable from that of untreated CD. The development of a so-called flat lesion was observed in approximately half of the patients (84–86). During the 1960's, a high incidence rate of cow's milk intolerance at infancy was found in diagnosed CD. Kuitunen et al. suggested that cow's milk intolerance may "pave the way" to CD (85).

A recent study by Hyytinen et al. did not find evidence that weaning off a diet of extensively hydrolyzed formula (compared to the implementation of cow's milk-based formula) would decrease the risk of CD later in life (87). It is not clear whether the breastfeeding and gluten introduction timelines presented in this study (87) are comparable with those from the previous, aforementioned studies (22–24). Furthermore, it is also currently unclear whether the cow's milk formula used by Hyytinen et al. (87) (80% intact milk protein, Enfamil; Mead Johnson, Chicago, IL, USA, and 20% hydrolyzed milk protein) mimicked the protein content and osmolarity of the homemade, cooked, diluted cow's milk used during the 1960s and early 1970s.

National Recommendations Causing Celiac Disease ("The Swedish Epidemic")

Celiac disease (CD) is an example of when a national recommendation can modify a disease's epidemiology. The trend in Europe of disappearing infant CD and the shift of age at diagnosis (19–24, 38) was not observed to be the same among all countries, with Sweden being an exception (40, 57, 77). In 2000, Ivarsson et al. investigated the countrywide epidemic of chronic malabsorptive syndrome occurring during infancy for a 10-year period from 1985 to 1995. The annual incidence rate of CD in children <2 years of age was found to increase four-fold during the epidemic in Sweden (40). Before the epidemic in 1983, recommendations for age at gluten introduction changed from 4 to 6 months of age, and as previously discussed, there was a rapid introduction of a high amount of gluten at the age of 6 months, inducing CD. In 1983, due to national recommendations, the amount of gluten in industrially produced weaning food in Sweden had been doubled on average. The curve presented by Ivarsson et al. for the wheat-rye-barley index based on the estimated average daily consumption of gluten-containing follow-on formulas followed the pattern of the epidemic in this country (40). However, it was observed that oats were not included in this cereal index curve but the estimated average daily consumption of oats was given (40). So, the curve for oats consumption was drawn exhibiting a U-shape, suggesting that the less oats ingested, the higher the incidence rate of infantile CD. The epidemic disappeared when wheat-based gluten content in the follow-on formulas was reduced (40). **Table 1** shows the differences in cereal consumption patterns for the ages of 9 and 12 months in Sweden and Finland.

An important message from the Swedish CD epidemic is that authority directives can completely change the overall makeup of a disease over a short time period.

Infections and the Microbiome

Infantile gastroenteritis and other infections could be potential environmental factors functioning at the small intestine mucosal level and potentially leading to overt CD in children exhibiting normal mucosa (62). Furthermore, the rotavirus has been implicated as potentially triggering the onset of CD (88). A large cohort study of children with HLA risk alleles for CD

found that frequent rotavirus infections (and not clinically symptomatic gastroenteritis) predicted a higher risk of CD autoimmunity (89). Furthermore, rotavirus infection increased CD autoimmunity, while vaccination seemed to reduce this risk (90). In fact, rotavirus infection may cause the manifestation of a small intestinal crypt hyperplastic injury with villous atrophy undistinguishable from the effects of gluten in CD, which was observed in both an experimental animal model and a child with rotavirus infection (91, 92). Recently, an experiment performed by Bouziat et al. on a human reassortant reovirus infection in mice was shown to trigger inflammatory responses to dietary antigens (93). In their study also CD patients tended to present with higher incidence rates of anti-reovirus (but not the rotavirus) antibody levels compared to the controls. Bouziat et al. further concluded that their study in humans supported the role of the reovirus, a seemingly innocuous virus, in triggering the development of CD (93). Recently, an association between enterovirus infection and later CD presentation was also reported (94). Furthermore, there has been evidence that, in general, any type of infection could be associated with an increased risk of CD (95, 96).

Since the rotavirus has been implicated as potentially triggering the onset of CD, live oral rotavirus vaccines could conceivably have a similar effect. However, a Finnish study in 2017 suggested that rotavirus vaccination did not increase the risk of CD in rotavirus-vaccinated children compared to those not vaccinated (97). In fact, rotavirus vaccination could even decrease the occurrence of CD in children and adolescents (98).

Tye-Din et al. discussed the complex role of microbiota in CD development (99). They found that factors such as environment, diet, and antibiotics can change the CD microbiome and its interactions with intestinal epithelial cells. The authors concluded that larger trials are warranted when microbiota are studied in at-risk individuals and must be followed-up with for a certain period of time to further understand gene-microbiome interactions in CD development (99). Recently, an observational, nationwide, register-based cohort study of children was performed in Norway and Denmark from 1995 to 2012 to elucidate the results of systemic antibiotic use in regard to CD (100), which indicated that childhood exposure to systemic antibiotics could be a risk factor of CD.

When examining the environment in which the changing pattern of CD occurred in children (**Figure 1**), robust epidemiological data supports the influence of gastroenteritis, especially of the bacterial variety rather than the rotavirus-induced disease (101). **Figure 3** presents all cases of childhood diarrhea in the country reported to the National Board of Health in Helsinki, Finland over two 10-year periods. Diarrhea cases occurring during the summer, which produced large infant mortality rates, were seen to completely disappear during the 1970s in Finland. Moreover, the shape of the curve was now typical of the rotavirus infection, with the rotavirus also associated with half of all hospitalization cases from the years 1977 to 1978 (102). Additionally, enteropathogenic bacteria accounted for only 10% of all hospitalized cases. These were Yersinia, Campylobacter, certain strains of Salmonella, a few enteropathogenic *Escherichia coli* serotypes (EPEC), and zero Shigellas (102, 103). It should be noted that classical cases of CD

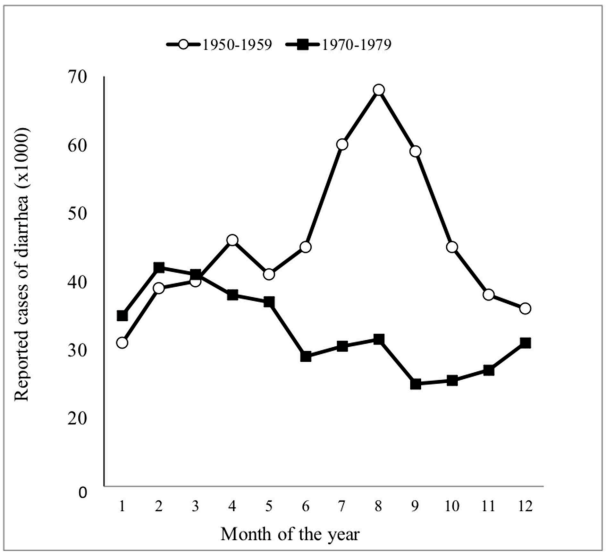

FIGURE 3 | Seasonal distribution of all reported cases of diarrhea in Finland during two 10-year periods [adapted from Mäki et al. (101)].

in young infants disappeared throughout Finland, even though the rotavirus was seen to greatly induce diarrhea and accounted for 50% of all hospitalization cases at that time (103). EPEC was seen to be most abundant during the summer months of the 1950s and was detected in 24% of all diarrhea cases in children ages 0–3 and in 28% of diarrhea cases for ages 3–6 months (101). Fewer "summer diarrhea" cases were reported during the 1960s; however, they were still quite prevalent and only completed to disappeared during the 1970s (101). Furthermore, the observed rate of hypertonic and hypotonic dehydration was only 3% in 1978, with 97% of all hospitalized children with diarrhea displaying isotonic dehydration (at the time when CD in infants had already disappeared) (101).

There are still some countries reporting the classical forms of CD in young children. For example, in Romania, enteropathogenic bacteria, EPEC, and infestations like giardia are all quite prevalent while very rare in Finland. Indeed, Romania is still currently experiencing several celiac crises, with eight new childhood cases occurring within just 1 year in Bucharest. On the other hand, general autoimmunity and pediatric chronic inflammatory bowel disease occur infrequently in Romania when compared to several other countries.

Autoimmunity and the Hygiene Theory

The increased prevalence of autoimmune diseases over time in industrialized countries is thought to be caused by a reduction in the incidence rate of infectious diseases, which is called the hygiene hypothesis. Early exposure to certain microorganisms during infancy directs and educates the human immune system. The frequency of infections directly contributes to an increase in autoimmune diseases, while infectious agents suppress autoimmune disorders (104, 105). Dysbiosis of commensal intestinal bacteria also plays an important role in autoimmune diseases, and the mechanisms inducing autoimmunity are a

complex combination of various environmental factors. In one study, intestinal microbiome development was examined in children from ages 0–3 in Northern Europe, in which early-onset autoimmune diseases are common, particularly in countries like Finland, while less common in countries like Russia (106). The study then suggested that the immunogenicity of early colonizing symbiotic bacteria could be a potential contributing factor to autoimmunity in humans.

The fact that diarrheal diseases and infections trigger CD and that antibiotic treatment at an early age increases the risk of the disease have already been mentioned in this paper. At the same time, the hygiene theory argues that the presence of microbiota and infections at an early age are essential in suppressing subsequent autoimmunity. CD is, in fact, an autoimmune disease in which, in contrast to other autoimmune diseases, the driving force behind the disease is known: the daily ingestion of gluten (107–110). Furthermore, a shift in the pattern of CD presentation toward older age at diagnosis has been evident (23), with the disease altogether not prevalent in adults prior to the 1970s. Changes in the clinical features of the disease in adults were examined in a study conducted in Scotland from 1960 to 79 (111) and later in Finland, with an increased awareness and use of autoantibody case-finding tools (8) associating with CD's high prevalence in adults (112). In certain population-representative screening studies, an increased prevalence of CD autoimmunity was noted by age: 1.5% in children (113), 2.0% in adults (114), and 2.7 % in the elderly (115). It was further shown that the prevalence of CD (i.e., autoimmunity) at the population level is increasing over time, doubling within the last two decades in Finland (114). Other studies later confirming this finding were conducted in the USA (116, 117). It has also been suggested that, for children, there is an increasing incidence over time (118, 119). Very recently, in fact, an increase in the prevalence in childhood CD was confirmed in Italy, showing a prevalence at the population level of 0.70% in 1994 and 1.58% in 2016 (120, 121).

In Russian Karelia, the population currently appears to be protected against autoimmune diseases. When compared to Finland, type I diabetes in children was six times less prevalent in Russia (122). A similar study comparing the prevalence of CD by screening 1988 schoolchildren from Russian Karelia and 3,654 children from Finland was performed and yielded the same result; CD was five times less frequent in Karelia. This may be associated with a protective environment characterized by inferior prosperity and standard of hygiene in Russian Karelia (123). In Finland, decreased microbial exposure in childhood seems to lead to increasing prevalence of autoimmune diseases and CD. In Russian Karelia, understanding could be enhanced in regard to the environmental factors involved in CD protection, as long as the sharp environmental gradient across the border is still existing. Vatanen et al. (106) have already initiated such studies on the microbiome.

INCIDENCE RATES AND PREVALENCE

In the 1980s in Tampere, Finland, an increase in the number of childhood CD cases diagnosed annually were reported at the same time as a decrease in incidence rates in both the UK and Ireland were also reported (19–22). As shown in **Figure 1**, this was due to a greater awareness of the mild form of the disease and atypical cases among school-age children. Additionally, by the late 1970s, the use of the modern tissue autoantibody test of the IgA class began to be used in case-finding, i.e., the R1 reticulin antibody test, later called the endomysial (EMA), and the tissue transglutaminase antibody (TG2-ab) test (12, 15). In 1990, it was reported that a decrease in the incidence rate of CD for children under 2 years of age occurs but that at school age increased significantly (83). This is displayed in **Table 2** where all children born in a strict geographical area during 1964–1988 were divided in 5-year birth cohorts and followed up to the age of 16. **Table 2** shows the numbers of CD patients diagnosed by the healthcare professional and the 5-year birth cohort incidence rate per year for different ages and the total prevalence during childhood up the age of 16. In the later birth cohort groups infant CD disappeared but the total prevalence of CD appeared stable during childhood (83). In terms of contracting the disease under 2 years of age, the risk for those born from 1964 to 1968 was 5.7-fold compared to those born from 1979 to 1983 or 1984 to 1988. Today, the children from the different birth cohorts are 30–55 years-old, and the current prevalence of CD among the same group

TABLE 2 | Birth cohort incidence rate of 1,000 live births per year at different ages and total prevalence of celiac disease during 5-year periods from 1964 to 1988 [adapted from reference Mäki and Holm (83)].

Year of birth (No. of live births)	Age of diagnosis (years)												Total prevalence (95% confidence interval)
	<2			2–5			6–10			11–16			
	n	Incidence	*p*[a]	*n*	Incidence	*p*[a]	*n*	Incidence	*p*[a]	*n*	Incidence	*p*[a]	
1964–1968 (29 056)	13	0.22		3	0.03		2	0.01		11	0.06		1.00 [0.64–1.36]
1969–1973 (24 646)	7	0.14	NS	3	0.03	NS	3	0.02	NS	8	0.05	NS	0.85 [0.49–1.22]
1974–1983 (25 962)	4	0.08	NS	3	0.03	NS	7	0.05	<0.10	10	(0.06)[b]	NS	(0.92)[b] [0.55–1.29]
1979–1983 (25 466)	2	0.04	<0.01	4	0.04	NS	9	(0.07)[b]	<0.025				
1984–1988 (25 500)	2	(0.04)[b]	<0.01	4	(0.04)[b]	NS							

[a] *Significance as compared to the incidence for birth cohort 1964–1968.*

[b] *Not final incidence and prevalence but at least minimum value.*

TABLE 3 | Changes in incidence rates of childhood celiac disease over time.

Author (reference number)	Study period	Age groups (years)	Incidence per 1,000 live births per year	Incidence per 100,000 person-years
Netherlands				
George et al. (125)	1976–1990		0.22	
George et al. (126)	1993–1994		0.54	
Steens et al. (44)	1993–2000		0.81	
Denmark				
Weile et al. (124)	1975–1990	0–18	0.10	
Michaelsen et al. (127)	1960–1988	0–18	0.09	
Dydensborg et al. (128)	1990–1999	0–18		0.8–1.4
Grode et al. (129)	1980–2016	0–9 (girls)		10.80
Italy				
Zingone et al. (130)	2011–2013	0–19		27.4
Greco et al. (39)	1975–1989		1	
Magazzu et al. (131)	1975–1989		3	
UK				
Greco et al. (39)	1975–1989		0.44	
Zingone et al. (132)	1993–2012			11.9
White et al. (118)	2005–2009			11.7
Sweden				
Greco et al. (39)	1975–1989		2.42	
Ivarsson et al. (40)	1984–1985	0–1.9		200–240
	1996	0–1.9		50
Tapsas et al. (133)	1994	0–1.9		
	2009	0–1.9		301
	2013	2–4.9		10
	2012	5–14.9		85
				78
Estonia				
Ress et al. (53)	1976–1980	0–19		0.10
	1986–1990			0.48
	1991–1995			1.55
	2001–2005			1.59
	2006–2010			3.14

could be evaluated, which is probably not the 0.1% shown in **Table 2** but rather 10–20 times higher, evidenced by population-based screening studies and the established pattern of increasing prevalence over time (113, 114). In addition, these birth cohorts, when followed-up with for 30–50 years, could further provide insights into this increase in prevalence over time. Thus, it is hypothesized that, based on the prevalence of the increase in population-representative adult materials (114), the two 5-year birth cohorts from the years 1964 to 1968 and 1969 to 1973 present lower total CD prevalence than the three 5-year birth cohorts from the years 1974 to 1988. However, it must be noted once more that, today, new seroconversions can still occur at older ages (115).

The lowest incidence rate per 1,000 live births (0.04) was reported in Estonia during the mid 1970s (78). In 2012, based on a nationwide study of childhood CD, Ress et al. showed a more than 30-fold increase in the incidence rate over a 35-year period in Estonia (53) (**Table 3**). This was due to an awareness of existing even clinically silent CD and case-finding produced through an autoantibody screening. Denmark and The Netherlands similarly reported very low incidence rates that increased over the years (**Table 3**), which are speculated to potentially be due to changes in the environment (124, 125). **Table 3** also shows the selected incidence rates of CD of several different countries per 1,000 live births every year or per 100,000 people every year.

Table 4 presents childhood CD prevalence figures, including both the biopsy-supported and serologically detected (celiac autoimmunity) diagnosis types, from various parts of the world. The overall prevalence of CD was observed to be ∼1%. However, population-representative screening further revealed that several countries, such as Argentina (135), Finland (113), Hungary (142, 143), Italy (121, 147–149), Spain (154), Sweden (157–160), and Turkey (163), were approaching a childhood prevalence of 1% or more. Meanwhile, countries exhibiting low CD prevalence included, among others, Estonia (138, 139), and Russia (123) (**Table 4**). The quite low prevalence rate observed for Denmark, even after its increase over the years (128), indicates that CD awareness is low among healthcare professionals and that serum autoantibody case-finding screening in the primary care field is not often performed. Furthermore, a questionnaire-based case-finding was conducted in Denmark in which children exhibiting celiac-related symptoms were invited to participate in a serological test. This evaluation showed that 14 out of 9,967 school-aged children suffer from CD in Denmark. For the same population, an additional 13 patients also had a

TABLE 4 | Prevalence of childhood celiac disease and celiac autoimmunity in different countries.

Author (reference number)	Study period	Prevalence biopsy proven (%)	95% Confidence interval	Prevalence serology based (%)
Australia				
Chin et al. (134)				0.6
Argentina				
Mora et al. (135)	2008–2009	1.30		
Brasil				
Pratesi et al. (136)	2003	0.54	0.27–0.57	
Egypt				
Abu-Zekry et al. (137)	2001–2004	0.53	0.17–0.89	
Denmark				
Dydensborg et al. (128)	1996–1999	0.04		
	2000–2010	0.08		
Estonia				
Ress et al. (138)	2004–2005	0.34	0.09–0.88	
Lillemae et al. (139)	1998–1999	0.34	0.09–0.88	0.43
Finland				
Mäki et al. (113)	1994	1	0.68–1.33	1.50
Germany				
Laass et al. (140)	2003–2006			0.8
Greece				
Karagiozoglou-Lampoudi et al. (141)	2009			0.65
Hungary				
Korponay-Szabo et al. (142)		1.20		
Korponay-Szabo et al. (143)	2005	1.38	0.94–1.82	
India				
Makharia et al. (144)	2008–2009	1.04	0.85–1.25	1.44
Iran				
Farahmand et al. (145)	2006–2008	0.5		
Dehghani et al. (146)		0.60		2.00
Italy				
Catassi et al. (120)	1994	0.70		1.5
Tomassini et al. (147)	1999–2000	0.94		
Mustalahti et al. (148)	1997–2000			1.1
Bonamico et al. (149)	2007	1.16		
Gatti et al. (121)	2016	1.58		
Netherlands				
Csizmadia et al. (150)	1987–1997	0.5		
Norway				
Størdal et al. (151)	2008–2011	0.38	0.37–0.39	
Russia				
Kondrashova et al. (123)	1997–2001	0.2		
Saudi Arabia				
Al–Hussaini et al. (152)	2014–2016			1.5
Spain				
Almazán et al. (153)	2009–2012			3
Cilleruelo et al. (154)	2004–2005	1.16	0.54–1.78	
Marine et al. (155)	2004–2007	0.49		
	2006–2007	0.49		
Castaño et al. (156)	1998–1999	0.84		
	after 2.5 years	1.4		
Sweden				
Carlsson et al. (157)	1995	2		
Carlsson et al. (158)	2000	1		0.7
Myleus et al. (159)	2005–2006	2.9	2.5–3.3	

(Continued)

TABLE 4 | Continued

Author (reference number)	Study period	Prevalence biopsy proven (%)	95% Confidence interval	Prevalence serology based (%)
Ivarsson et al. (160)	2005–2006	2.90		
	2009–2010	2.20		
Tunisia				
Hariz et al. (161)	2003–2005	0.45		0.64
Hariz et al. (162)	2011	0.24		
Turkey				
Demirceken et al. (163)		0.9		
Dalgic et al. (164)	2006–2008	0.47	0.38–0.57	1.74
United Kindom				
Mustalahti et al. (148)	2000			0.9
USA				
Fasano et al. (165)	1996–2001	0.31	0.09–0.80	

previous diagnosis of CD; thus, the prevalence rate increased to 0.27% (166). However, this value is still quite low for a country in which screened type 1 diabetes patients are known to often conduct CD as well (10.4%) (167). The high prevalence rate (3%) of CD in Swedish children also can be commented upon (159). In that study in Sweden, the children were born during the epidemic described, experienced high volumes of gluten in their weaning food, and their screening took place when they were 12 years of age. It can be speculated that their high gluten intake at a young age contributed to the 3% prevalence rate. On the other hand, it should also be noted that in the Swedish study the diagnostic criteria for CD were somewhat unconventional, also including symptomatic patients with only increased intraepithelial lymphocyte counts shown in their biopsy results (Marsh class 1). Patients with no symptoms but exhibiting Marsh 1 lesions were again not considered to have CD. Overall, when comparing the incidence and prevalence studies from different countries in which a biopsy was the primary detection method, the poor celiac center pathologist interobserver reproducibility of the small intestinal mucosal injury, i.e., the Marsh classes, must be kept in mind. A normal mucosa graded as Marsh 0 may be Marsh 3 c and vice versa (168, 169).

CONCLUSIONS

The observed changes in childhood CD presentation and epidemiology have been remarkable during the past 50 years, with overall clinical childhood CD presenting as a malabsorption syndrome transforming into a milder form, resulting in an upward shift of age at diagnosis. The incidence rates and prevalence of childhood CD have been constantly increasing due to case-finding screening strategy. The most important factor in the increasing rates of CD seemed to be an awareness of healthcare professionals of this changing pattern, the existence of which is quite evidence-based. This change took place at different times in different countries, resulting in several global differences. Today, the best case-finding biomarkers for the screening of undiagnosed CD are gluten-dependent serum autoantibody tests, and it is recommended that these tools be used in the primary care field. There is also evidence of an increase in CD prevalence in children over time that is similar to that observed in adults. The main question involved in this change in prevalence asks for the reasons behind these observed clinical changes. This study thus discusses changing environmental factors, such as breastfeeding, gluten, and cow's milk ingestion, and infections as causes for the observed changes. To prospectively readdress these questions using modern epidemiological tools, it is suggested that studies be conducted in countries in which the classic presentation of CD in young children, including diarrhea and malabsorption syndrome, still exists. Overall, an oral tolerance toward gluten could still exist in certain settings, and the environmental factors involved in infantile CD and increasing prevalence of autoimmunity at older ages could still be further investigated by future studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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To Be Oats or Not to Be? An Update on the Ongoing Debate on Oats for Patients With Celiac Disease

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To date, the only known effective treatment for celiac disease (CD) is a strict gluten-free diet (GFD) for life. Patients with CD often find it difficult to adhere to strict GFD. Oats, compared with wheat, barley, and rye, contain less amounts of prolamins. Inclusion of oats in a GFD might be valuable due to their nutritional and health benefits and increase of food variety. Therefore, they may potentially improve feeding diversity for these children and improve taste and satiety. We reviewed the literature to evaluate the safety of oats in CD patients. We have searched PUBMED, societal guidelines and national health authorities' recommendations. The following aspects were reviewed: gastrointestinal symptoms, malabsorption, serology including specific avenin antibodies, mucosal changes, avenin toxicity, immunogenicity of oats, and quality of life. We also referred to wheat contamination of oat products, the safe amount of oats for CD patients and the type of oats recommended. Data support that pure oats are well-tolerated by most CD patients, at moderate amounts (20–25 g/day dry rolled oats for children; 50–70 g/day for adults). Nevertheless, since the potential for sensitivity/toxicity exists, oats should be added with caution to a GFD, only after all CD symptoms including weight loss and growth disturbances have resolved, after at least 6 months of conventional GFD and probably also after normalization of serology. The need for pre exposure biopsy is unclear and should be considered on an individual basis.

Keywords: oats, celiac, safety, adherence, small bowel

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INTRODUCTION

Children with celiac disease (CD) often find it difficult to adhere to a strict gluten free diet (GFD) (1). Oats, compared with wheat, barley and rye, contain less amounts of prolamins (10 vs. 33–50%) (2), which are the gluten components responsible for the toxicity noted in susceptible individuals. Therefore, inclusion of oats in a GFD may potentially improve feeding diversity for these children (1), and also improve taste and satiety (3).

The GFD may be low in fiber, iron, folate, calcium, magnesium, zinc, and B-complex vitamins (thiamin, riboflavin, niacin, and vitamin B12), as well as vitamin D. Kilned and unkilned varieties of oats can improve vitamin B1, magnesium, and zinc intake in patients with CD in remission, and thus provide a significantly better nutritional profile compared to regular GFD (4). Oats also represent a good source of fiber in a GFD. Betaglucan in oats lowers postprandial plasma glucose and attenuates insulin responses. In addition, it increases bile acid excretion and transport with subsequent lowering of low-density lipoproteins. Oats contain about twice as much protein as

rice. Oats also contain 6–8% of oil with a high proportion of unsaturated fatty acids. In addition, avenanthramides (specific antioxidants) are also present in oats (4).

Since Dicke's studies many decades ago (5, 6) there has been a controversy over the inclusion of oats in GFD. Early studies on patients with CD suggested intestinal malabsorption and exacerbation of abdominal symptoms after ingestion of oats (7, 8). More recent *in vitro* and *in vivo* studies have re-questioned the toxicity of oats and have suggested that inclusion of oats in a GFD may be reasonable and safe. This report considers the current evidence for oats in individuals with CD.

GASTROINTESTINAL SYMPTOMS FOLLOWING INGESTION OF OATS

Baker and Read (8) measured urinary excretion of an oral 5 g xylose load before and 4 weeks after an oatflakes challenge in 11 adult patients and one child with CD on GFD for at least 6 months. They noted that six patients developed either gastrointestinal (GI) symptoms or anorexia and irritability; three of them had reduction in xylose excretion to between 47 and 77% of the pre-challenge levels.

Peräaho et al. (9) randomized 39 patients with CD on a GFD without oats who had documented mucosal recovery (though not complete in all cases) either to consume 30 g of oats-containing gluten-free products daily or to continue their regular diet without oats. The follow-up time was 1 year. They observed a trend toward a higher Gastrointestinal Symptom Rating Scale (GSRS) in the oats group, and the symptoms of diarrhea were more severe in the oats group at the end of the study (statistically significant). The constipation score increased similarly in both groups, while indigestion symptoms were improved in both groups, but more effectively in patients taking oats.

Koskinen et al. (10) enrolled 23 children with CD (ages 7–18 years) who maintained a conventional GFD (including abstinence of oats) for at least 2 years. Thirteen children were then randomized to undergo an open oats challenge and 10 had a gluten challenge, allowing the consumption of wheat, rye, and barley in addition to oats. Median daily oats consumption was 45 g/day. During the 2 years trial two children who ingested oats, but not gluten, developed dramatic GI symptoms but without signs of immune activation or CD relapse on small bowel biopsies.

Størsrud et al. (11) followed 20 adults with CD in remission who were taking a daily intake of 100 g of uncontaminated rolled oats for a period of 2 years. Flatulence was the most pronounced reported symptom achieving maximum intensity at 6 months.

Although these studies showed symptomatology in patients with CD exposed to oats, this is not the case in other studies. For example, Janatuinen et al. (12) followed 52 adult patients with CD in remission and 40 patients with newly diagnosed CD for a period of 12 months. They divided each group into two: those consuming oats and those who did not. The mean oat intake in the oat group was 45 g daily. They found that the use of oats by adult patients with CD in remission as part of a GFD had no unfavorable effects, and did not prevent symptomatic healing in patients with newly diagnosed disease.

Furthermore, Gatti et al. (13) in their 15 months double-blind, randomized, placebo-controlled multicenter study, enrolled 306 children with CD who had not previously consumed oats. They were randomized into two groups following either A-B treatment (6 months of diet "A," 3 months of standard GFD, 6 months of diet "B"), or B-A treatment (6 months of diet "B," 3 months of standard GFD, 6 months of diet "A"). A and B diets included gluten-free (GF) products with either purified oats or placebo, respectively. They monitored GI symptoms, growth data, and intestinal permeability tests (IPT) with measurement of urinary lactulose/mannitol (L/M) ratio. They found that the prolonged intake of a considerable amount of daily oats did not cause any change in clinical symptoms or intestinal permeability. Nevertheless, the specific amount of oats ingested was not documented in this study.

Sey et al. (14) challenged 15 adults with CD with 350 g/week of pure, uncontaminated oats for 12 weeks. The patients had been asymptomatic on a GFD for at least 1 year and had a normal tissue transglutaminase (tTG) level at the entry to the trial. They were largely asymptomatic throughout the study: there were no significant changes in mean pain, diarrhea, flatulence, or abdominal distension scores. In a separate study conducted by Hardman et al. (15), none of the 10 patients with dermatitis herpetiformis reported pruritus, rash, GI symptoms, or other adverse effects during a 12 weeks exposure to purified oats.

MALABSORPTION

Lindsay and Moulton (7) fed four children with CD a GFD apart from the inclusion of quick porridge oat flakes. The oat flakes intake was between 46 and 169 g per day for a period of 22–96 days. Although high oat flakes intake increased fecal fat excretion, it was still within normal limits.

Tjellström et al. (16) analyzed fecal short chain fatty acids (SCFA) concentration in 116 children with newly diagnosed symptomatic CD for a 1 year period. Fifty seven of them were consuming oats containing GFD, and 59 were on a standard GFD. The SCFA patterns found in the fecal samples represent the unabsorbed fraction of SCFAs produced in the GI tract. The researchers noticed high concentrations of the pro-inflammatory acetic acid and total SCFA throughout the diet period in the GFD-oats group. This is in contrast to a significant decrease in total SCFA concentration in the GFD-standard group. No correlation was noticed between the amount of oats consumed and SCFA levels. The clinical implications of this finding are unclear. Albeit this finding, all children were in clinical and histological remission at the end of the study (except for one child in the GFD-standard group, who did not undergo a control biopsy).

As opposed to the former studies, Srinivasan et al. (17) demonstrated that lactase expression, which is an indicator of small bowel insult, was undetectable in nine patients with untreated CD, lost in patients who underwent a gluten "micro

challenge," and was normal in those on a strict GFD. Oats challenge, on the other hand, did not affect lactase activity.

SEROLOGICAL RESPONSES TO INCLUSION OF OATS

Sey et al. (14) challenged 15 adults with CD with 350 g/week of pure, uncontaminated oats and found no serological relapses of tTG after 12 weeks of challenge. Cooper et al. (18) showed no change as well in the level of mucosal tTG and smooth muscle alpha actin expression in small bowel biopsy tissue of patients with CD after a 1 year period of oats ingestion in addition to a GFD. Furthermore, Hardman et al. (15) detected no antigliadin (IgA and IgG), antireticulin (IgA), or antiendomysial (IgA) antibodies in patients with dermatitis herpetiformis before or 12 weeks after a purified oats challenge.

Koskinen et al. (10) enrolled 23 children with CD into either an oat (45 g/day) or gluten challenge (14 g/day) in addition to oats for a period of 2 years. They showed no change in the intensity of small-bowel mucosal TG2-specific autoantibody deposits with oats ingestion. However, when wheat, rye, and barley were consumed in addition to oats, a marked small-bowel mucosal antibody response occurred in parallel with small-bowel mucosal damage within 3–12 months. When an oat-containing GFD was adopted after relapse, the small-bowel mucosal IgA deposits intensity significantly decreased within 6 months, indicating that these histological changes occurred secondary to the other cereals.

Størsrud et al. (11) reported that a large daily intake of uncontaminated rolled oats during a 2 years period did not cause any negative serological effects in adults with CD in remission. Furthermore, Janatuinen et al. (19) showed that oats did not prevent normalization of gliadin and reticulin antibodies in new onset CD nor did they cause any significant changes in gliadin antibody levels in CD patients in remission.

Picarelli et al. (20) showed that no endomysial antibodies (EMAs) were detected after 72 h of culturing CD duodenal biopsy specimens with a peptic-tryptic digest (PT) avenin. In contrast, EMAs were detected in all 13 samples after 72 h of an *in vitro* challenge with PT gliadin.

Högberg et al. (21) enrolled 116 children with newly diagnosed symptomatic CD in a randomized, double blind, multicenter study. The children were divided into two groups to receive a standard GFD with or without pure oats and were followed for a period of 12 months. The median amount of oats ingested in the GFD-oats group was 15 g daily. Ninety-two patients completed the study. There were no differences in anti-gliadin antibodies between the GFD-oats and GFD-standard groups.

SPECIFIC AVENIN ANTIBODIES

Guttormsen et al. (22) investigated IgA antibodies against wheat gliadins, oats avenin and tTG levels in three groups of individuals: patients with CD on a GFD with ($n = 54$) or without oats ($n = 82$) and healthy individuals ($n = 141$). Both CD patient groups had equally elevated antibodies to oats compared with healthy subjects. Hvatum et al. (23) showed that patients with untreated CD have not only antibodies to wheat and oats, but also to a range of different food antigens (soy, milk proteins, and ovalbumin). Accordingly, Guttormsen et al. (22) assumed that the ongoing wheat-specific T-cell immune response affects all mucosal B cells and that the high anti-avenin antibodies in CD patients does not support a harmful effect of oats. It is worth noting that when Hvatum et al. (23) examined antibodies to different food antigens in patients with CD, they compared them to patients with various other intestinal disorders and not to a healthy population.

Hollén et al. (24) found that children with CD had developed both IgG and IgA avenin antibodies on gluten challenge. These levels positively correlated with those against gliadin and dropped to the reference level on GFD. To note, they did not challenge the patients with oats. In a later study, the same group performed serial assessments of anti-avenin antibodies in 116 children with CD on a GFD with or without 15 g (median) daily oats consumption (25). They concluded that avenin alone is not able to initiate the immune response.

MUCOSAL CHANGES FOLLOWING OATS EXPOSURE

Janatuinen et al. (12) found no worsening of the villous architecture after 6 months of oats exposure in patients with CD in remission. In patients with newly diagnosed disease, both groups (oat consuming and GFD only) achieved similar mucosal remission. Even a longer follow-up (5 years) showed that oats ingestion does not result in any duodenal mucosal damage in adults with CD (26). In a later study they found no difference in the densities of CD3+, αβ+ intraepithelial lymphocytes (IELs), and γδ+ IELs between those consuming oats and those on a conventional GFD (27).

Koskinen et al. (10) showed that during a 2 years trial, oats had no detrimental effect on intestinal mucosal villous morphology, densities of CD3+, αβ+, and γδ+ IELs or HLADR celiac expression. Further, Högberg et al. (21) in their 1 year study mentioned above, did not find significant differences in small bowel biopsies between the GFD-oats and GFD-standard groups. The two children with an abnormal mucosa were in the GFD-standard group.

Sey et al. (14) found no significant changes in symptoms, biochemistry, or histology after oat challenge in 15 adults with CD. One patient's biopsy worsened from modified Marsh 1 to 2, likely because of non-compliance with the GFD. Another patient had a change from modified Marsh 3a to 3b.

Hardman et al. (15) examined 10 adults with dermatitis herpetiformis who consumed 50–70 g of gluten free oats daily for 12 weeks. They found no evidence of any abnormality of the villous architecture, including depth of crypts or duodenal intraepithelial lymphocytes.

In addition, in a study performed by Størsrud et al. (11) there were no changes in the villous architecture or grade of inflammation in adults with CD in remission who were taking a daily intake of 100 g of uncontaminated rolled oats for a 2 years period.

Janatuinen et al. (19) showed that oats did not prevent normalization of the number of IELs in the duodenal mucosa of newly diagnosed patients nor do they cause any significant changes in the number of IELs in CD patients in remission.

Holm et al. (28) challenged 23 children with CD in remission with either oats (median daily consumption 43–45 g/day) or gluten (median daily consumption 14 g/day). They found that after 2 years of consuming oats, all had normal densities of αβ+ IELs. In contrast, small bowel mucosal deterioration was evident within 3–12 months in all CD patients randomized to a gluten-challenge.

Kaukinen et al. (29) followed 110 adults with CD treated for up to 8 years. They found that the mucosal morphology was significantly better in subjects who had consumed larger amounts of oats or had a longer oats exposure compared to those who did not consume oats.

In contrast to the above studies, others have reported mucosal architecture damage in patients with CD consuming oats. Tuire et al. (30) followed 177 adults with CD adhering to a strict GFD for at least 2 years. They observed that persistent intraepithelial lymphocytosis was more common in long-term treated patients (42%) than in the general population (4%). At a cutoff value of 30 IEL per 100 enterocytes, oats consumption was the only factor that contributed to the persistent small-intestinal mucosal inflammation. However, at a lower cutoff value of 25 IELs per 100 enterocytes, no association between oat consumption and IEL was found.

In a study conducted by Peräaho et al. (9), the density of IEL was significantly higher in the oats consuming group, but the change in GSRS was not associated with the increase in IEL density.

Lundin et al. (31) reported a patient who showed deterioration in her small bowel histology (from Marsh type 1 to Marsh 3A lesion) during a 12 weeks challenge with 50 g of oats daily. These changes were accompanied with slight dermatitis and positive IFN-c mRNA levels. The mucosal changes resolved back to Marsh 1 after 12 weeks of a gluten and oats free diet, but recurred to Marsh 3B after a further 8 weeks oat re-challenge, accompanied with diarrhea, clinical dermatitis herpetiformis, and increased levels of IFN-c mRNA. Her mucosa failed to recover even 1 year after the re-challenge (Marsh 3A lesion). The purity of oats was tested by ELISA kit of antibody against v-gliadin, which detects barley to lesser extent. To note, this patient was one of 18 CD patients who were included in the study, whose small intestinal biopsies showed either unchanged or improved histology following oat challenge. Twelve patients who continued to consume oats beyond the challenge period had Marsh type 0–1 in their biopsies after 1.5 years of consuming oats.

AVENIN TOXICITY

Real et al. (32) showed that avenins have a lower proline content compared to wheat gliadins and low molecular weight (LMW) glutenin subunits. The proline content positively correlated with the toxicity of the storage proteins of various cereals. Therefore, avenins have lower celiac toxicity with respect to wheat prolamins. In this study, however, the researchers also showed that some oat varieties could be potentially immunotoxic.

Mujico et al. (33) observed reproducible differences in the gamma-gliadin reactive T cell (which also respond to avenin peptides) stimulatory capacity of 26 oat samples. These cells are isolated from the small intestine of patients with CD and thus are strongly linked with CD. They concluded that most non-contaminated oat varieties contain avenin epitopes that are potentially harmful for a minority of the CD patient population.

Arentz-Hansen et al. (34) also demonstrated responses to TG2-treated avenin in polyclonal T-cell lines derived from the avenin-challenged biopsies from nine patients with CD, three of whom had clinical and histopathological signs of oat intolerance.

Kilmartin et al. (35) demonstrated similar immunoreactivity of CD mucosal T cell lines to protein fractions from wheat cereals, barley, rye, and oats. However, they also showed that despite their T cell line stimulation, oats do not activate a mucosal lesion in most CD patients.

IMMUNOGENICITY OF OATS

Some studies have found that oats are not immunogenic in patients with CD.

In an *in vitro* study conducted by Kilmartin et al. (36) avenin did not trigger an IFN-γ mRNA, nor did it induce significant IL-2 mRNA production in eight duodenal biopsies. This was in contrast to nine tissue samples following 4 h of culture with gliadin, where a significant increase in IFN-γ mRNA production and in IL-2 mRNA was observed.

Another *in vitro* study found that a 1 year oats challenge in patients with CD adhering to a GFD caused no change in CD8+ T cells number in the small bowel epithelium, which is a classic active CD lesion (18). In addition, oats did not alter the extent of enterocyte Ki67 expression, which reflects increased cell turnover.

Tapsas et al. (37) measured the urinary nitrite/nitrate excretion in 188 children and adolescents with CD. Increased secretion of proinflammatory cytokines and NO metabolites precedes the mucosal alterations, thus it is considered a reliable indicator of small intestinal inflammation. There was no correlation between urinary NO metabolites and oats consumption or duration in the GFD.

Srinivasan et al. (38) examined expression of immunological molecules, considered to reflect immune activation in small bowel tissue, of 10 CD patients taking 50 g of oats daily for 12 weeks. They found no increase in major histocompatibility complex (MHC) class II molecule, CD25 positive cells, the intercellular adhesion molecule 1 (ICAM-1), the nuclear proliferating antigen Ki-67 or mast cell enzyme tryptase expression. Moreover, MHC class II staining developed and numbers of CD25 positive cells increased when the patients were given a gluten challenge.

Hardy et al. (39) assessed whether oats ingestion or other known toxic grains in CD stimulate an avenin-specific T cell

response *in vivo*. They fed participants oatmeal (100 g/day over 3 days) to measure the *in vivo* polyclonal avenin-specific T cell responses to peptides contained within comprehensive avenin peptide libraries in 73 HLA-DQ2.5(+) patients with CD. Grain cross-reactivity was investigated using an oral challenge with wheat, barley, and rye. Avenin-specific responses against four closely related peptides were observed in six (8%) of the 73 subjects.

Sjöberg et al. (40) studied the same group of patients that Högberg et al. (21) had enrolled and analyzed changes in mRNAs expression levels of for immune effector molecules and tight junction proteins in small intestinal biopsies from patients treated with GFD+oats and standard GFD, respectively, before and after the intervention. The researchers found that mRNA levels for all five chemokines tested (CX3CL1, CXCL8/IL-8, CXCL9, CXCL10, and CXCL11), followed disease activity with no difference between the two GFD diets. On the other hand, when they tested proinflammatory and downregulatory cytokines, they found that mRNA for IL-10, TGF-β1, and TNF-α, did not normalize in several patients on oats-containing GFD. Regarding NK receptors and MHC class I molecules, KLRC2/NKG2C and KLRC3/NKG2E mRNA levels normalization did not occur to the same extent on the GFD+oats diet as on the GFD-std diet. Some individuals even showed increased levels. As far as for tight junction proteins, CLDN-4 mRNA levels were higher in active CD than in controls and showed significant decline after GFD-std but not after GFD+oats. The researchers concluded that there was ongoing immune activity in the intestinal mucosa of patients in the GFD+oats group. More studies implied the presence of immunogenicity in some varieties of avenins.

The mucosal level of IFN-c mRNA in treated CD patients on a standard gluten and oats free diet is usually below 1×10^3 transcripts per μg total RNA. Lundin et al. (31) found that after an oats provocation period, five of 19 patients with CD had detectable levels of IFN-c mRNA. One of these five also had a positive level before the provocation, and one patient was tested only afterwards.

Silano et al. (3) demonstrated that avenins were able to activate peripheral lymphocytes from patients with CD, although avenins from different cultivars displayed different immunogenic activity. In another study, this group indicated that some varieties of oats may be potentially harmful to individuals with CD (41). The differences may be partially explained by the fact that oat immunogenicity depends on the cultivar used.

Comino et al. (42) showed that there are differences in immunogenicity of various oat varieties, and that some of them are not reactive against the T cells of patients with CD. They reported the G12 antibody as competent to identify potentially toxic oat varieties for patients with CD. In another study, though, this group found the existence of new potentially toxic peptides. These peptides were able to activate circulating dendritic cells (the most potent antigen-presenting cells of the immune system that have the capacity to trigger T-cell proliferative responses from patients with CD) (43).

QUALITY OF LIFE

In a 2 years intervention study conducted by Størsrud et al. (44), 15 adults with CD consumed an average of 93 g of rolled oats daily. All patients commented on the beneficial effects of oats, i.e., a better taste, the satiating effect, more variability and improved bowel function. These effects were independent of the amount of oats consumed.

Peraaho et al. (9) found that oats had no effect on quality of life (QOL) as measured by the Psychological General Well-Being (PGWB) questionnaire. In another study, this group surveyed 494 patients with CD who were on an oat-containing GFD: 94% of them felt that oats diversified their GFD and was in many respects beneficial (45).

Aaltonen et al. (46) followed a large cohort of 869 patients with CD for a 1 year period. They demonstrated that patients with CD consuming oats(all aged over 16 years) as part of a longstanding GFD had similar or even somewhat better QOL than those not consuming oats. Further, there was no difference between the groups in symptoms, CD serology, and small-bowel mucosal damage after 1 year on a GFD.

WHEAT CONTAMINATION OF OAT PRODUCTS

The fact that oats are often processed on the same production line as wheat raises the possibility of gluten contamination. Gimenez et al. (47) examined 132 oat accessions and showed that 73% of them could be considered "gluten-free" (in the range 3–20 ppm). Mujico et al. (33) in contrast to the previous group observed that commercially ground oat flours were mostly contaminated with other cereals.

A study conducted in Spain analyzed 108 oat samples (e.g., rolled oats, oat flakes, and flours) collected from Europe, the United States, and Canada (48). Three quarters of the samples were contaminated with more than 20 ppm of gluten: with variation of up to 8,000 ppm.

In the USA, 9 out of 12 containers of rolled or steel-cut oats, representing four different lots of each of three separate brands (Quaker, Country Choice, and McCann's) had gluten levels from 23 to 1,807 ppm. All three brands of oats had gluten levels above 20 ppm (49).

HOW MUCH OATS IS GOOD ENOUGH?

The tolerance threshold for gliadin has been evaluated to be 20 ppm (50–53). The daily intake of oats is considered to be 40–80 g, corresponding to 0.2–1.2 g avenin, which is about 10% of the gliadin toxicity amount. On that basis it can be speculated that a much higher amount (about 10 times) than the average daily intake of oats is required to induce a toxic mucosal reaction (2).

A Health Canada publication recommended that the amount of uncontaminated oats consumed by individuals with CD should be limited to 20–25 g/day for children and 50–70 g/day for adults (1).

WHAT KIND OF OATS?

A study conducted by Kemppainen et al. (54), in which 33 patients with CD consumed more than 90 g daily of either kilned or unkilned oats for 12 months, demonstrated that adding high amount of kilned oats into the GFD led to increased vitamin B1 intake.

According to the American College of Gastroenterology (ACG) clinical guidelines, oats should be introduced into the diet with caution and patients should be monitored closely for evidence of any adverse reaction (strong recommendation) (55). In addition, according to the ESPGHAN clinical guideline, unless the purity of the oats can be guaranteed, their safety remains questionable (56).

SUMMARY

A large number of reviews have been published on the matter of the safety of oats in GFD. Most of these reviews concluded that pure oats are well-tolerated by most CD patients (4, 55, 57–61), at moderate amounts (20–25 g/day dry rolled oats for children; 50–70 g/day for adults) (1, 62) or even up to 100 g/day (63). Inclusion of oats in a GFD might be valuable due to their nutritional and health benefits and improvement of food variety (64, 65). Nevertheless, since the potential for sensitivity/toxicity exists (2, 58), oats should be added with caution to a GFD (55, 57), only after all CD symptoms including weight loss and growth disturbances have resolved (1), after at least 6 months of a conventional GFD (1, 4, 66) and, in our opinion, also only after normalization of serology. The need for pre-exposure biopsy is unclear and should be considered on an individual basis. These patients should be closely monitored for evidence of intolerance (1, 4, 55, 66) and some even suggest annual screening biopsies to ensure that subclinical changes are not occurring in the small bowel mucosa (57). Oats contamination by gluten must always be considered (4, 56, 63, 67), mandating a continuous need to ensure oats purity (56, 60, 67, 68).

AUTHOR CONTRIBUTIONS

IS reviewed the literature and wrote the manuscript. RS reviewed the literature and helped with the writing of the manuscript. AD reviewed the manuscript and helped with the writing of the manuscript.

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REVIEW
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Navigating the Gluten-Free Boom: The Dark Side of Gluten Free Diet

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In gluten dependent conditions the gluten free diet is the cornerstone of therapy, decreasing disease activity, improving health and quality of life and treating or preventing the associated complications. Gluten withdrawal implies strict and lifelong elimination not only of wheat, barley, rye, and wheat-contaminated oats, but also of numerous non-nutritional products where components of wheat are often added. Due to multiple reasons the diet is difficult to follow and the long-term adherence is decreased with time. The present review summarizes the dark side of gluten restriction where nutritional deficiencies, toxicity, morbidity, mortality, and mental health problems are reported. The aim being to increase awareness, avoid, detect and treat the side effects and to promote a healthier nutrition, for the patient's benefits.

Keywords: celiac disease, gluten free diet, gluten withdrawal, side effects, nutritional deficiency, non-celiac wheat sensitivity, morbidity, mortality

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INTRODUCTION

The global population has more than doubled in the last 40 years supported by the *"green revolution"* in agriculture producing high-yield grain varieties, including semi-dwarf, high-yield, disease resistant varieties of wheat, that are central to the modern diet (1). Awarding the Nobel Peace Prize in 1970 to Norman Ernest Borlaug recognized the value of dwarf wheat to humanity.

Celiac disease has traditionally been clinically considered and then investigated with patients presenting with gastrointestinal (GI) symptoms. However, for every adverse reaction to wheat presenting with GI symptoms, there are 8 presenting without GI symptoms (2). Thus, dependence on GI complaints as a prerequisite in considering an adverse reaction to wheat, will allow a majority to escape diagnosis. This is a critical point of recognition for the Clinician when considering an association of wheat related disorders (WRD) and the potential value of a Gluten-Free Diet (GFD).

The 8:1 ratio of extra-intestinal vs. intestinal symptoms is not limited to celiac disease. In a prospective 1-year study of suspected non-celiac gluten sensitivity (NCGS) related disorders from 38 Italian centers—all recognized as referral centers of excellence and included in the register of the Italian Health Ministry for the diagnosis of gluten-related disorders, 53% of patients presented with non-abdominal complaints. The most frequent extraintestinal manifestations were fatigue and lack of well-being, reported by 64 and 68%, respectively, of the enrolled subjects. In addition, a high prevalence of neuropsychiatric symptoms including headache (54%), anxiety (39%), *"foggy mind"* (38%), and arm/leg numbness (32%) were recorded. Other extra intestinal manifestations emerging from the analysis of the survey responses were joint/muscle pain often misdiagnosed as fibromyalgia (31%), weight loss (25%), anemia (22%)—due both to iron deficiency and low folic acid, depression (18%), dermatitis (18%), and skin rash (29%), Umberto et al. (3). With its global impact in the body and lack of isolated tissue vulnerability, a high degree of suspicion is required for a Clinician to investigate a presenting patient for a WRD.

GFD, the mainstay of treatment for celiac disease (CD), is increasingly being adopted by people without a diagnosis of celiac disease. Gluten-free (GF) eating patterns have become a mainstream phenomenon during recent years, and nearly one-third of Americans report having attempted to eliminate or reduce the amount of dietary gluten they consume (4).

Currently, wheat is the most widely cultivated crop in the world and the dominant staple crop in temperate countries, providing between 20 and 50% of the total calories intake. The gluten family of proteins are the major proteins of wheat and are very important for wheat survival. In industrial countries, wheat makes substantial contribution to diet and health, particularly providing dietary fibers, B vitamins (B1, B2, B3, B6, B9) and mineral micronutrients, notably iron, zinc, and selenium. Hence, restricting wheat intake, without the guided support of a well-trained Registered Dietician, Nutritionist, or Physician can have serious consequences for the intake of essential nutrients and other beneficial components. GF menus are significantly lower in protein, magnesium, potassium, vitamin E, folate, and sodium, with suggestive trends toward lower calcium and higher fat (4). Equivalent sources of essential nutrients must be provided.

A common misnomer is that GF substitute foods are healthy. There is no evidence to support such a claim. While it is true that GF foods will eliminate a primary antigenic component of the diet, GF foods are calorie rich and nutrient poor. Restricting the intake of wheat in the diet can have serious consequences for the intake of essential nutrients and other beneficial components unless equivalent sources of these are provided (5). It is generally considered that GF foods are less nutritionally adequate than standard products. GF bread products were significantly higher in fat and fiber. All GF products were lower in protein than standard products. Only 5% of GF breads were fortified with all four mandatory fortification nutrients (calcium, iron, niacin, and thiamin), only 9% of GF bread products were fortified with thiamin, riboflavin, and niacin, and 28% of GF breads were fortified with calcium and iron only. This lack of fortification may increase the risk of micronutrient deficiency in celiac sufferers.

Based on the modern awareness on the nutrition-health bidirectional connections, the topic of healthy food is today's fashionista. According to the World Health Organization, a healthy food should prevent under and over nutrition and protect from chronic, non-communicable diseases (6). Controversially and in real facing of the mirror, the metabolic syndrome, allergy, cancer, stroke and autoimmune conditions are surging in the last decades (7–9). All 5 of these conditions have inflammation as the igniting spark associated with their development. Mirroring those alarming surges with simultaneous substantial declines in infectious diseases set the stage for the hygiene hypothesis. However, new scientific winds are blowing and "cleaning" the hygiene related concepts and prioritize the friendly microbiota over the pathogenic dysbiota in shaping gut homeostasis (10, 11). Changes in the microbiome on a GFD have substantial influence on the pro/dysbiotic environment of the GI Tract. It appears that intimate evolutionarily relationships are important, indispensable and crucial for bugs and us to develop and survive (12–14). We need them and they can't survive without our gut hospitality. Here comes the major environmental factor that keeps the physiological microbiome at bay: healthy, well-balanced food. Many food intolerances and allergies, metabolic conditions, genetic abnormalities, autoimmune diseases, and other medical conditions require special or controlled diets, GFD being one of them. Due to the incomplete adoption of a GFD outside the boundaries of a proven gluten related disease, the present review will concentrate on the dark side of gluten withdrawal.

Abbreviations: CD, celiac disease; GF, gluten free; GFD, gluten free diet; GI, gastrointestinal; NCGS, non-celiac gluten sensitivity; WRD, wheat related disorders.

Gluten Dependent Conditions

Several diseases are considered to be gluten dependent. Gluten allergy that follows immune allergic mechanisms, celiac disease (CD), gluten ataxia and dermatitis herpetiformis as autoimmune conditions and the recently describe entity: non-celiac gluten/wheat sensitivity (15, 16). Celiac disease is a lifelong autoimmune disease affecting genetically predisposed individuals by the consumption of prolamins like wheat, barley, rye, and contaminated oats (17). It affects averagely 1–1.5% of Western populations, its incidence is surging worldwide even in far-east and under developed countries, a trend shared by many other autoimmune diseases (8, 18). Co-emergence of increased gluten consumption and CD prevalence and its recent changing epidemiology, reinforce the environmental over genetic influence in the contemporary CD surge. The only proven therapy is a lifelong GFD that reverse most of the symptoms, improve substantially mucosal pathology, near to the normal histology and prevent some of the long term complications. GFD can improve the other gluten dependent conditions. Prevent the allergy, alleviate the skin eruption in dermatitis herpetiformis, and potentially improve the ataxia in gluten ataxia. Despite being an effective therapy, multiple difficulties arise during its implementation.

Gluten Free Diet

The only recommended and effective therapy for CD is strict exclusion of gluten for life. Despite strict adherence, complete mucosal recovery is rarely obtained. In most cases, a variable degree of inflammation persists characterized by persistent intraepithelial lymphocytosis with or without associated glandular hyperplasia (Marsh I-II/Grade A) at control biopsy usually performed after 6–18 months of GFD. Complete normalization to Marsh 0 stage was observed in 8% of patients, with Marsh I and II lesions persisting in 65% of patients with duodenal atrophy at baseline (19). And in the largest study ever done on Mortality and Celiac Disease, of 46,121 total biopsies, Marsh I-II was identified in 13,306 specimens. This was associated with a 72% increased risk of mortality in the first year after diagnosis (20). This is almost double the increased mortality in the first year after diagnosis with total villous atrophy CD (39%). The persistent duodenal intraepithelial lymphocytosis in CD patients during GFD is not eliminated by a gluten contamination elimination diet and is independent of the time length of GFD (21). GFD include withdrawal of

wheat, barley, rye, contaminated oats, spelt, kamut, triticale, malt, or their hybridized strains. Natural gluten free nutrients like vegetables, legumes, fruits, fish or unprocessed meat, milk and dairy products, and eggs are allowed. By various industrial nutritional technics, gluten can be eliminated or decreased to <20 ppm, the amount allowed, for the CD patients by the European codex alimentarius (22). At least for now, GFD is fully recommended for CD, gluten allergy, dermatitis herpetiformis, and gluten ataxia. For the newly evolved entity of non-celiac gluten sensitivity, GFD might alleviate their symptoms, though, the pathophysiological pathways, offending environmental factors, strict diagnostic criteria and therapeutic nutrient withdrawal policy, are far from being resolved (23–25). The diagnostic criteria for CD and its related extra-intestinal manifestations (26, 27) are quite clear and the recommended gluten avoidance is obvious, but the lifelong restriction is a bumpy road (28, 29). The difficulties in following GFD and the reasons for poor patient's compliance were summarized recently (28, 29). Gluten is considered as a universal food additive as a thickener, flavor enhancer, filler, emulsifier, or texture modifier in many processed food products (6, 30). The present review will zoom on the deleterious effects of GFD, but, before will describe the nowadays fashionista of gluten/wheat avoidance.

GLUTEN WITHDRAWAL FASHIONISTA

In parallel to the surge in gluten-related disorders prevalence, the numbers of people that empirically try to avoid gluten, for a variety of signs, reasons, and symptoms are gradually and continuously rising.

It becomes a very popular diet in contemporary history. Although many report improved health, increased energy, social and food industries and several best-selling books fuel its popularity, there is little evidence to explain the above mentioned reports. The opponents to and the approvers of gluten came to the forefront of popular nutritional discussions and "going gluten-free" has become mainstream in Western populations (31, 32). Currently, ∼25% of the population reports keeping a GFD, many without adequate knowledge "requesting GF meals and ordering bread pudding for dessert" (33, 34). On the other hand adherence to GFD by the gluten dependent patients is poor and deserve continuous nutritional education and behavioral reinforcement programs (35, 36). No doubt, gluten restriction became today's fashion trend (37).

Recognizing that dietary changes alter the gut microbiome in humans within 24–48 h (38), a 2011 study demonstrated that for celiac children following a GFD for 2 years, the GFD did not completely restore the microbiota and, consequently, the metabolome of CD children (39). In another study, 10 healthy participants who did not have CD or a recognized gluten sensitivity, following a GFD, found that their diet alone created a pro-inflammatory GI environment 100% of the time. Researchers concluded that when individuals discontinue eating wheat, the reduced polysaccharide intake (prebiotics) creates a shift in the diversity of the intestinal microbiome, with lowered *bifidobacterium* and *lactobacillus* and a concomitant increase in *enterobacteriaceae*, diminishing short chain fatty and organic acids and thus reducing competence of the immunomodulatory role of the microbiome with lowered TNFα, IFN gamma, IL-8, and IL-10. This outcome creates a pro-inflammatory environment (40).

With wheat providing 78%, and barley (3%), together providing 81% of oligo fructose and inulin for average North Americans (41), a non-guided GFD, which does not purposefully replace these critical fructan prebiotics (such as Arabinoxylose), runs the risk of creating a starvation state for the resident probiotics, easily creating an inflammatory cascade environment in the microbiome. Without these fructan prebiotics in wheat, the microbiota which was accustomed to this family of prebiotics die off, creating a pro-inflammatory environment (42).

An example of the potential complication of the above dynamic is the production of trimethylamine N-oxide. This metabolite of choline increases atherosclerotic plaque size, triggers prothrombotic platelet function, promotes arterial thrombus growth (43), and predicts risk for cardiovascular diseases (44). This is produced by action of genera *Allobaculum* and *Candidatus arthromitus* as well as the family *Lachnospiraceae, which* were identified in the cecal microbiota producing higher levels of serum trimethylamine N-oxide (45). Hence, the composition of the commensal microbiota is an emerging risk factor for cardiovascular diseases.

In view of the rising popularity of gluten restricted products, the present review will summarize the dark side of the GFD, rather than describing the macro and micronutrients deficiencies, extensively described in naïve or untreated CD patients (46).

NUTRITIONAL DEFICIENCIES

GFD is an unbalanced diet that buries in its lap multiple nutritional deficiencies. It is known that gluten restricted products are of poorer nutritional value, lower quality, unpleasant mouth-feel, less flavored and diminished viscoelasticity of bread products. **Table 1** summarizes those deficiencies while being treated with GFD. The fact that most of the GFD products are not fortified, further aggravates the deficiencies. Summarizing surveys on nutritional profiles of gluten free, compared to gluten containing food products, Melini et al. showed that the GFD is deficient in fibers, proteins, folate, iron, potassium, and zinc, while higher in fat, carbohydrate, sugars, FODMAP's, and sodium, in most of the surveys (6).

The substitution of gluten containing prolamins by rice and corn put the patient at risk of protein, fiber and folate deficiencies (50, 60) and higher glycemic index (60). Thus, partially explaining the higher rate of metabolic syndrome and cardiovascular morbidity reported in naïve and treated CD patients (61–64). One must remember the association of an activated immune response to other foods in over 50% of celiacs maintaining an inflammatory environment in the gut (65).

TOXICITY

There are two main sources for toxic or potentially deleterious compounds, while consuming GFD. The first one is due to the common consumption of fish and rice. Both have increased levels

TABLE 1 | Nutritional deficiencies, excesses and toxicity in gluten free products.

Nutrient abnormality/deficiency	Excessed components	Toxicity
Iron	Proteins	Heavy metals: arsenic, mercury, lead, cadmium
Calcium, sodium	Fat, saturated fatty acids	Food additives: enzymes like microbial transglutaminase, proteases
Vitamin D	Sugar, sucrose	
Vitamin C	Energy intake	
Vitamin A, E		
B Vitamins: B12, thiamin, riboflavin, niacin	Fat, saturated fatty acids	
Folate	High Omega 6 fatty acids	
Zinc, Magnesium, Selenium	Higher arachidonic acid: docosahexaenoic acid ratio	
Fibers: oligo fructose, inulin, fructans	Higher pro-inflammatory fatty acid profile	
Low HDL, Apo A1		
Lower essential amino acids		
Low arachidonic acid: dihomo-γ-linolenic acid ratio		

Adapted from Taetzsch et al. (4), Melini and Melini (6), Lerner et al. (13), Lerner and Matthias (30), Dennis et al. (46), Penagini et al. (47), Shepherd and Gibson (48), Theethira and Dennis (49), Hosseini et al. (50), Freeman (51), Lerner and Matthias (52), Matthias et al. (53), Matthias and Lerner (54), Lerner and Matthias (55), Allen and Orfila (56), Riezzo et al. (57), Lamacchia et al. (58), and van Hees et al. (59).

of heavy metals like lead, cadmium, mercury, and arsenic [(51), **Table 1**]. In a recent study, persons following GFD, including non-celiac patients, had increased urinary levels of arsenic and blood levels of mercury, lead, and cadmium compared to persons consuming gluten (66). Since most of the GFD consumers today are non-celiac people, those results relay the accumulation of the toxic heavy metals to the gluten restrictive diet, rather than to CD (67). An additional reason to avoid the gluten-free "fashionistic" fad.

The second potential source of toxic harmful compounds is the enzymes used as food additives in the processed food gluten free products [(50), **Table 1**]. Microbial transglutaminase (mTg) imitates functionally the autoantigen of CD, namely, the tissue transglutaminase (52). It is a very common additive for cross linking proteins, thus, changing the physical properties, 3D structure, and immunogenicity by exposing new epitopes, and food quality of many GF products, including in the bakeries (50, 52, 53). Most recently, the enzyme was suggested as an environmental factor that drive CD development (52–55). Its potential deleterious effects in GFD, deserve further exploration.

INCREASED MORBIDITY AND MORTALITY

Undiagnosed or untreated CD patients are at risk for acute (68) or chronic morbid manifestations and associated diseases.

TABLE 2 | Summary of the causes of increased morbidity on GFD.

Increased morbidity	References
Social leisure activities	(32, 50)
Metabolic syndrome	(64)
Hepatic steatosis	(64)
Cardiovascular risks	(4, 62, 63)
Depression	(56)
Diabetes 2	(4)
Osteopenia/ osteoporosis	(69)
Reduced beneficial microbiota	(42, 70)
Loss of around 80% of prebiotics	(42)
Obesity	(4, 71)

However, when compliant to GFD the risk for morbidity and early mortality continues. The background might be inherent to GFD content or to their continued intestinal inflammation. **Table 2** summarizes the morbid conditions that CD population on gluten restricted diet might risk.

The increased mortality in CD populations was previously reported. In the largest survey of mortality in CD, mortality was increased in CD, in latent CD and in patients with intestinal inflammation, hazard ratio (HR) 1.39, 1.35, and 1.72, respectively (20).

The HRs for mortality were highest in the first year of follow-up after diagnosis with total villous atrophy CD associated with a 2.80-fold increased risk of death, latent (positive serology, negative histology) celiac disease with a 1.81-fold, and inflammation (increased intraepithelial lymphocytes) with a 4.66-fold increase. These startling statistics should bring pause to every Clinician. The only difference post diagnosis for the vast majority of newly diagnosed celiacs is implementation of the GFD. With maintaining inflammation in the intestines on a GFD, the HR for mortality in the first year post-diagnosis was almost double that of total villous atrophy CD. The necessity of comprehensive education for transitioning the patient to a healthy GFD, free from emphasis on GF commercial foods high in calories and low in nutrients, cannot be overemphasized. The most common cause of death in the first year post diagnosis was cardiovascular diseases, followed by malignancy. Excess mortality was independent of the intestinal damage and was observed even on GFD, most probably due to the persistent mucosal inflammation. In fact, after an average of 12 years on a GFD, 31% of patients still have increased enteric inflammation (72). Suicide rate is increased in CD, latent CD and persistent intestinal inflammation with HR of 1.55, 1.06, 1.96, respectively, compared to the general population, when done on a Swedish cohort (73). Interestingly but alarming, CD diagnosed in childhood was associated with a 40% increase in suicide risk. It should be noticed that naïve and treated CD is frequently comorbid with multiple additional autoimmune diseases like autoimmune thyroiditis, type 1 diabetes, IBD and many more (14, 26, 27, 74). Cancers like non-Hodgkin lymphoma and other gastrointestinal malignancies and genetic conditions like Downs, Turner, and Williams syndromes are also associated with CD,

thus adding to the morbidity and mortality, including when on gluten withdrawal. At the end of the day, it is possible that persistent inflammation increased both overall mortality rate and suicide risk, despite following GFD.

GFD AND MENTAL HEALTH

Evidence points to the nervous system as the prime site of gluten damage. Literature reported that gluten can cause neurological harm through a combination of cross reacting antibodies, immune complex disease, and direct toxicity (75). The association between CD and other gluten related conditions with behavioral and psychiatric disorders was extensively reported and summarized recently (76–78). The spectrum includes anxiety, dysthymia, depression, attention-deficit hyperactive disorders, mood and sleep disorders, mal social adaptation, substance-related, addictive and neurocognitive disorders, suicide attempts, learning disabilities, feeding and eating disorders, bipolar syndromes, schizophrenia, and autistic spectrum disorders. Those disorders appeared across different age groups, in both genders and some are considered as extra-intestinal manifestation of CD. The strongest association was reported with depression (59, 76), but, the association between chronic medical conditions and depression is well-established. It might be bidirectional since physical illness or disability often worsen the mental disorder and vice versa (79). More so, dietary modification, including GFD in gluten related conditions, may be associated with pathological eating practices (80). Compliance with GFD is a tough alley (28), associated with major changes in daily lifestyle routine and activities and eating habits, many times stressful and difficult to accept (81, 82). The underlying mechanisms that induce those psycho-social-behavioral disorders, while on GFD, are not understood. Nutritional and vitamin deficiencies (59), an immune dysregulation, cerebral hypo perfusion (83), the gut "feeling of the brain" in the gut-brain axis (84) or the stress of adherence to GFD, have been suggested (76).

GFD SIDE EFFECTS: CLOSING THE GAPS

Taking into account the tough alley and the contemporary torrid times in complying with a strict GFD (28), sincere and practical efforts should be implemented to close the gaps. Following are some suggestions to avoid the detrimental effect of GF product consumption toward a more personalized nutrition, tailored to the individual needs.

Gluten restriction should be accompanied by healthier food to avoid nutritional deficiencies, toxicity of alternative non-gluten containing prolamins, morbidity and finally early mortality. In this regards, it is advised to adapt the Mediterranean diet, the most studied and successful one against major non-communicable chronic diseases (85). It is rich in fibers, anti-oxidants, anti-inflammatory mediators, vitamins, trace elements, minerals, beneficial fatty acids, qualified proteins, bone building compounds, metabolically balanced and affects the enteric microbiome favorably (86, 87). Enriched cow's milk and dairy products are salutogenic to CD patients and their lactose intolerance is transitory while on gluten restriction (88, 89). If a patient has neither clinical nor serology evidence of a sensitivity to cow's milk (90), and they are not homozygous for a primary lactase deficiency (91), the nutrient dense platform of milk may be of substantial value in supplying macro and micro nutrients for rebuilding an inflammatory-damaged terrain.

Asking a patient to abstain from a most-common food in their diet, often eaten in some form multiple times per day, is an extremely challenging directive fraught with non-compliance absent expert on-going advice. Early and effective consultation with a gluten knowledgeable, food labeling skilled, Dietitian, Nutritionist or Health Coach (28), increasing the intake of pseudo-cereals, polyphenol-rich fruits and vegetables, encouraging natural GF foods, focused efforts on rebuilding a balanced non-inflammatory microbiome, increasing the patient's education on the disease evolvement, GF products and follow ups, in order to improve adherence and establish a healthy balanced diet (6, 47, 49, 50, 92) is of essential nature in reducing the increased risk of morbidity and mortality. Very important is the physician-dietitian-patient trustful relationship and the team communication for the patient's successful recovery and adherence to the dietary restriction. Not less important is the periodic laboratory test to check for CD associated serological markers and to detect any nutritional deficiencies.

CONCLUSIONS

GFD is the only proved therapy for CD and is crucial in the other gluten related conditions. It reduces activating an inflammatory cascade in the intestines, rehabilitates the intestine, normalizes the associated biomarkers and can treat or improve some of the extra-intestinal manifestations. However, despite being an effective therapy, GF products suffer from limitations, side effects and might bear risks if not detected and dealt appropriately. Nutritional deficiencies, toxic components, unbalanced diet, increased morbidity, mortality and psychiatric or behavioral abnormality, are a few examples. The treating team should be aware of the potential dark side of gluten restriction, diagnose those abnormalities and offer adequate solutions to improve the patient's health and quality of life.

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AL and TO'B: conceptualization. TO'B: methodology. AL: formal analysis. TM: resources. AL and TO'B: data curation. AL: writing—original draft preparation. AL, TO'B, and TM: writing—review and editing. All authors read and approved the final manuscript.

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