

# DIET AND CELIAC DISEASE

From disease risk to management

JOHANNA KREUTZ



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## **Colofon**

Diet and celiac disease: From disease risk to management

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**DIET AND CELIAC DISEASE**  
From disease risk to management

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*Dedicated to my Oma Paula,  
who was denied further education beyond primary school and ensured that for future  
generations of women doors of opportunity were opened wide.  
And who also taught me the art of cheating at card games.*



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# GENERAL INTRODUCTION

## **“Does this have gluten in it?”**

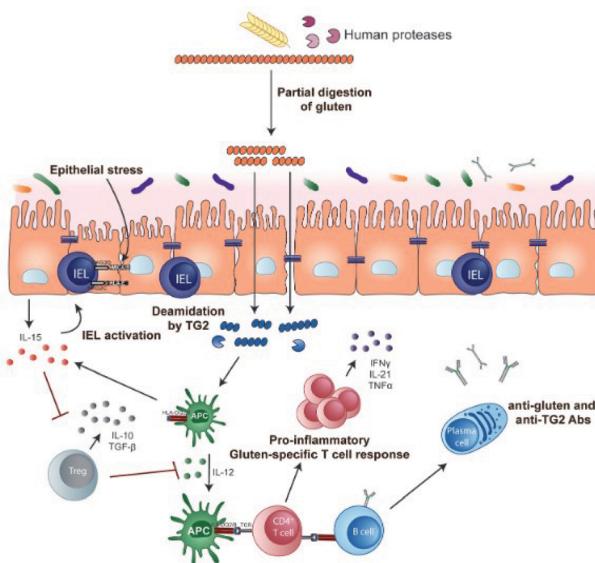
Millions of patients with celiac disease (CD) ask the same question on a daily basis: “does this product contain gluten?” CD is a chronic disease affecting 1-2 % of the population in Europe and North America (1-5). The prevalence of CD in Africa and Asia is not well studied, but appears to be about 1% as well (5-8). In CD, ingestion of gluten triggers an auto-immune response that leads to damage to the small intestinal mucosa and subsequent intestinal- and extra-intestinal symptoms, including abdominal pain and bloating, diarrhea, fatigue, weight loss and nutrient deficiencies (9, 10).

The only current treatment for CD is a lifelong strict gluten-free diet (GFD). The challenges of a GFD together with the occurrence of CD comorbidities lead to a high perceived disease burden in both children and adults with CD (11-15). This calls for further strategies to reduce the risk of new onset of CD on the one hand and improvement of disease management on the other hand.

## **Celiac Disease Etiology**

CD results from an aberrant response of the adaptive immune system to peptides derived from gluten in genetically predisposed individuals (Figure 1). The gliadin peptides of gluten cross the intestinal mucosa, are deamidated by tissue transglutaminase (tTG) and are presented by antigen presenting cells with HLA-DQ2 or HLA-DQ8 cell surface receptors. This activates a CD4+ T cell response, including release of pro-inflammatory cytokines and other mediators. Together with recruited natural killer cells, these mediators result in damage to the intestinal wall of the small intestine, which is distinctive for CD. The villous atrophy and crypt hyperplasia lead to malabsorption and the resulting possible consequences include among others iron deficiency anemia (IDA) ((16-20); chapter 4,5).

Next to T cell activation, B cells are activated and produce IgA and IgG antibodies against gluten peptides and against the autoantigen tTG, which are thought to contribute to extra-intestinal manifestations of CD such as dermatitis herpetiformis (10, 21, 22).



**Figure 1.** Key steps in CD pathogenesis. Adapted from: Tye-Din et al., *Front Pediatr.* 2018.

It has been well established that exposure to gluten in combination with a genetic susceptibility, more specifically positivity for HLA DQ 2 and/or HLA DQ 8 genotype, are two important risk factors for CD to develop (10, 21, 23-25). However, those two factors alone do not entirely explain the onset of CD, as the frequency of HLA DQ2/8 in the general Western population is 40%, while only 2% develop CD and 98% of the carriers do not (10, 21, 25, 26). Thus, other external and internal risk factors must therefore be at play. The identification of risk factors, particularly modifiable ones, is highly relevant to identify targets for disease prevention. Potential and known risk factors include non-HLA genes (such as IL-2, IL-21, CCR3), female gender, (viral) infections in early life, shifts in gut microbiota composition, social economic status, and dietary risk factors including timing of gluten introduction and amount of gluten ingestion in early life as well as the Western Diet (27-33).

Circumstantial evidence suggests that intestinal barrier disruption, is not only a hallmark of CD, but may play a crucial role in the development of the disease as well. An increased intestinal permeability with altered TJ proteins has been shown in CD (34-38). A dysfunctional barrier, or leaky gut, is hypothesized to be an important facilitator of gliadin peptides crossing the intestinal barrier into the lamina propria where it can be processed into its highly immunogenic components, which in turn come in contact with the immune cells. Increased presentation of deamidated gliadin to T cells located along the intestinal wall is thought to correlate with the increased occurrence of the aforementioned aberrant immune response.

The small intestinal barrier consists of the gut microbiota, a mucus layer, lymphoid cells and the epithelial monolayer characterized by the intercellular junctional complex forming a selective barrier that facilitates the absorption of nutrients and water while limiting the entrance of microbes and antigens (39, 40). The epithelial layer is made up of specialized cells for absorption called enterocytes, as well as various secretory cells including the enteroendocrine cells, Paneth cells and Goblet cells. In order to maximize the absorptive capacity of the small intestine, the epithelium is organized in a villous structure and enterocytes have microvilli yielding a large absorptive surface.

The inter-cellular epithelial space is sealed by the junctional complex comprising adherence junctions, desmosomes and most importantly tight junctions. These comprise e.g. cytoplasmic (actin-binding) proteins and transmembrane proteins (39, 40). The distribution and expression levels of these molecules and junctions can be altered in response to various triggers such as pro-inflammatory cytokines (e.g. IL-1 $\beta$ , IL-6, TNF $\alpha$ , and IFN- $\gamma$ ), microbial infections (e.g. LPS, bacterial toxins, and viral infections) as well as dietary factors (41, 42).

High intake of gluten, as a component of the Western diet, has been proposed as risk factor for CD. Gliadin exposure has shown to increase intestinal permeability and may itself therefore form a possible barrier disruptor and risk factor as well (43, 44). Additionally, a high intake of processed food and sugars have been identified as risk factors for CD development, but may also impair the intestinal barrier (27, 45, 46).

The role of these Western diet components in pro-inflammatory processes and disease development has been explored in other chronic diseases rising in prevalence, such as metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD)(47-49). High sugar intake therefore may be an interesting modifiable factor to reduce the risk of CD development.

## **Celiac Disease Management In Clinical Practice**

The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) published extensive guidelines for the diagnosis of CD in children. The most recent iteration, published in 2020, includes options for pediatricians to diagnose CD without an invasive endoscopic procedure, which was required in all previous guidelines 2012 (9, 50). The most important tool to diagnose CD is serological testing of CD specific auto-antibodies (i.e. anti-tissue transglutaminase [anti tTG] and anti-endomysium antibodies [anti EMA]). Analysis of these

biomarkers can be combined if necessary with clinical symptoms, histopathological examination of small intestinal biopsies and HLA typing to establish the diagnosis (9, 50). However, advancements in understanding the pathophysiology of CD have also highlighted the potential role of intestinal fatty-acid-binding proteins (I-FABP) for both CD diagnosis and management. FABPs are intracellular transporters that are highly abundant in small intestinal enterocytes and have shown to be a useful marker for enterocyte damage (51, 52). Recent studies have demonstrated that I-FABP has the potential to serve as a biomarker for intestinal damage in CD, making it potentially useful in diagnosis and follow-up of intestinal recovery, but also for investigating intestinal damage prior to disease onset (53-55).

After CD diagnosis, for children in particular, a lifelong journey of disease management starts. This includes the challenges of following a strict, lifelong GFD, as well as tackling other aspects of the disease burden, such as regular check-ups with blood testing (56, 57).

How pediatricians should monitor and manage CD, is however not well established in National or International guidelines, mainly due to lack of evidence for a specific approach. This was also highlighted in a recent position paper by ESPGHAN, which provided expert consensus statements on CD management (58). For instance, they noted that an important component is the follow up of nutrient status. Patients with CD are at risk to develop nutrient deficiencies due to several important reasons. First, the CD distinctive intestinal damage can result in malabsorption and nutrient deficiencies, which can persist for some time after a GFD has started (59, 60). Second, the GFD itself forms a risk for nutrient deficiencies, as it is a challenge to maintain a balanced diet with sufficient macro and micronutrients. Gluten-free grains are usually less dense in micronutrients (i.e. iron and folate) than their gluten containing counterparts. Further, gluten-free products often are less nutrient dense and contain more sugar, salt and fat in order to improve taste. Maintaining a GFD therefore not only requires vigilance in avoiding intake of even the smallest amount of gluten, but also for monitoring the overall quality of dietary intake. Nevertheless, how these attempts to control food intake translate into clinical benefits, e.g. reduced risk of developing nutrient deficiencies, still remains unclear, because of the lack of dedicated studies. This impedes the development of evidence-based recommendations on how and with which frequency nutrient status should be monitored in children with CD.

Recently, the COVID-19 pandemic brought a new challenge for both patients with CD and their caregivers, adding to the uncertainties experienced by all children. The pandemic might have placed an extra burden on CD patients and their families, who require specialized healthcare access and specific dietary products. The decrease in food security and availability amplified concerns for those following

medical diets. Government-imposed regulations, including social distancing and homeschooling, created a natural experiment by controlling external factors for populations in multiple countries. A natural experiment is an observational study in which an event or situation occurs naturally and is exploited to answer particular questions. This unique opportunity also allowed for investigating the impact of the pandemic on general health and perceived disease burden of CD patients, such as potential limitations in accessing healthcare and in maintaining a GFD. Additionally, exploring the experiences of children with CD and their families during this time could provide valuable insights for CD management in general, including lessons from the utilization of telehealth services and changes in eating and shopping behaviors.

## Aims And Outline Of This Thesis

This thesis has two main aims with the overarching goal of reducing disease burden of CD in children from infancy through adulthood. The first aim was to investigate the role of dietary factors and barrier disruption in CD etiology (see chapters 2&3). The second aim was to examine challenges of the GFD beyond the elimination of gluten (see chapters 4 to 6).

In **chapter 2**, the presence of small intestinal damage prior to CD onset was explored in a unique European birth cohort of individuals with a high risk to develop CD. Presence of intestinal damage early in life was assessed using I-FABP, measured in serum of these high risk individuals, that have been followed up for up to 16 years after birth.

The Western diet has been identified as a possible modulator in barrier disruption and risk factor for CD development. In **chapter 3**, the effect of dietary simple carbohydrates, an important component of the Western Diet, on intestinal barrier function was assessed using a well-established *in vitro* cell culture model of Caco-2 monolayers.

The occurrence of nutrient deficiencies in patients with CD was examined in **chapters 4 and 5**. First, in **chapter 4**, the scientific literature was reviewed on the evidence of nutrient deficiencies in adult and pediatric patients with CD. Additionally, the different reasons for possible nutrient deficiencies, as well as their specific possible clinical consequences in CD were reviewed. In **chapter 5**, the occurrence of aforementioned nutrient deficiencies was evaluated in a patient cohort of pediatric patients treated at the MosaKids children's hospital of the Maastricht UMC+. The data from this electronic chart review is comprised of a cohort of patients with a follow-period of up to 10 years.

In **chapter 6**, challenges of the GFD were explored in a survey study of pediatric patients with CD and their caregivers during the first wave of the COVID-19 pandemic. The unique developments during the beginning of the pandemic in the Netherlands were used as a natural experiment to learn more about experiences and perceived challenges of children with CD and their families concerning the disease itself and the GFD in particular.

Finally, in **chapter 7** the main outcomes of the thesis are discussed and summarized and recommendations for further research and clinical application are given.

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# I-FABP CHANGES IN EARLY LIFE IN INFANTS AT RISK TO DEVELOP CELIAC DISEASE

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## **Abstract**

### **Background**

Intestinal mucosal damage is a hallmark of coeliac disease (CD) and is hypothesized to be important in CD etiology. Circulating intestinal fatty acid binding protein (I-FABP) concentration is a marker for enterocyte damage in the small intestine. By measuring serum I-FABP, we evaluated whether mucosal damage is present very early in life and prior to CD seroconversion in children with a high risk to develop CD.

### **Methods**

I-FABP concentration was measured in consecutive serum samples very early in life of children with a high risk to develop CD, from the European birth cohort PreventCD. I-FABP was compared between 61 children who developed CD (CD group) and 76 controls that did not (nonCD). A post hoc analysis was conducted in a subgroup of 27 children who developed CD before 3 years of age and the nonCD group. I-FABP was compared with pooled samples at different age categories and using linear mixed models.

### **Results**

In the samples taken between 7 and 9 months of age, I-FABP concentration was significantly higher in the children who developed CD before the age of 3 years compared to the nonCD group ( $p=0,019$ ). The linear mixed models analysis showed a significant difference in I-FABP between CD and nonCD group. No significant difference in I-FABP concentration and development over time was found in the total group.

### **Discussion and conclusion**

This study showed a marked difference in the course of I-FABP concentration over time in the first year of life in a specific subgroup, namely between children who develop CD at an early age (<3 years) and those who didn't develop CD, in a group of European children with a high risk to develop CD. This effect was not seen, when including children in the analysis that developed CD after 3 years of age. Intestinal barrier alterations early in life could be either indicators of or risk factors for CD development during childhood in individuals with a high risk to develop CD based on genetic susceptibility and family history. The results of this study warrant a more in depth investigation of gut maturation, functionality and damage early in life and its influence on risk to develop CD.

## 1. Introduction

The understanding of the pathological mechanism and clinical management of celiac disease (CD) has largely improved in the last years (1, 2). However, successful primary preventive strategies for CD have not been identified, as we lack knowledge on risk factors and their mechanism of action in disease onset (3). Consequently, research efforts are shifting towards this field, with the ultimate goal being the prevention of this chronic condition (4). This would have a significant impact on the health and well-being of affected individuals and their families.

The current body of evidence suggests, that several features recognized as part of the manifestation of CD are also present in a more subtle form prior to disease onset. Among these features, altered growth velocity, immune response, circulating miRNA expression and gut microbiota composition are notable examples (5-8). Altered growth for instance, albeit not to the severe extent of failure to thrive often displayed at diagnosis, is already observed before disease onset, in children at risk to develop CD (8).

It remains unclear, whether these features in early childhood are signals of disease manifestation, or risk factors contributing to disease onset.

Besides impaired growth and an altered immune response, intestinal epithelial damage is a hallmark of active CD. This raises the question, whether intestinal damage is a feature present prior to disease onset as well. Circumstantial evidence suggests that intestinal function is altered prior to CD onset, as shown in CD animal models (9). Additionally, healthy first degree relatives of patients with CD show altered intestinal permeability compared to low-risk controls that are not carriers of the HLA-DQ2 and 8 alleles (10).

We hypothesize that intestinal barrier damage may contribute to the development of CD by enabling a greater amount of gluten and pathogens to cross the barrier, which can trigger an immune response (11, 12). This in turn may increase the likelihood of the altered immune response associated with CD to occur in genetically predisposed individuals. Understanding the role of intestinal barrier damage in CD development is crucial for comprehending disease pathogenesis and identifying potential targets for prevention.

The objective of this study was to map the course of intestinal damage in children who are at high risk to develop CD very early in life, prior to disease onset.

For this, we studied intestinal damage in the PreventCD cohort, a European birth cohort investigating the risk factors for CD prior to its onset. Subjects were at high

risk to develop CD based on genetic predisposition (HLA DQ 2 and/or 8 positivity) and family history for CD. They were followed prospectively with predefined, regular blood sampling. Intestinal damage was assessed by utilizing the biomarker intestinal fatty acid binding protein (I-FABP), a sensitive marker for small intestinal epithelial damage.

## **2. Methods**

### *Subjects and sample collection*

Serum samples from the European birth cohort, PreventCD were utilized for I-FABP concentration measurement (13). In short, PreventCD followed 944 genetically predisposed children from birth and presence of at least one first degree relative with confirmed CD diagnosis. Follow-up included regular blood sampling (during the first 3 year seven-times with predefined intervals), and an array of outcomes such as health complaints by standardized questionnaires, anthropometrics, and gluten-intake assessment. In case of suspicion for CD, the diagnosis was confirmed following ESPGHAN guidelines (14, 15).

In a subgroup of this cohort, I-FABP concentration was measured in a selection of 2-4 samples per subject. The subject selection was based on sample availability and disease status. We aimed to include two blood samples within the first 12 months of life if available. All samples were taken prior to seroconversion. The moment of seroconversion was defined as the first blood sample in which CD autoantibodies were measured, usually IgA anti tissue transglutaminase (anti tTG).

### *Primary and secondary outcomes*

The primary outcome was I-FABP concentration at every measurement moment, as well as disease status. Subjects were divided into two groups, children who developed CD during follow-up (CD group) and children who have not developed CD until moment of locking the data (nonCD group). The dataset was locked for analysis on 20.06.2023, taking disease status of study subjects up until that moment into account. At moment of locking the dataset, the youngest participant in this study was 12 years and the oldest 16 years old.

Further sex and HLA genotype were noted and HLA genotype was categorized into four risk groups. HLA risk groups were defined as: group 1: DQ2.5/DQ2.5 and DQ2.5/DQ2.2; group 2 DQ2.2/DQ7; group 3 DQ2.5/DQ7, DQ2.5/DQ8, and DQ2.5/other; group 4 DQ2.2/DQ2.2, DQ2.2/DQ8, and DQ8/DQ8; DQ2.2/other, DQ8/DQ7, and DQ8/other. Further, celiac disease antibody concentrations were noted per measurement moment, if they were measured and available. For all subjects in the CD group, moment of seroconversion was noted.

### I-FABP concentration measurement

Blood serum samples were stored at -80C and thawed for analysis in batch. I-FABP concentration was measured in serum in duplicate with an enzyme-linked immunosorbent assay (ELISA). The assay was conducted at Maastricht University in The Netherlands, according to the instructions of the manufacturer (R&D systems-DY3078 DuoSet ELISA for FABP2). The absorbance of the samples was measured using a spectrophotometer. The coefficient of variation (CV) was calculated between the duplicates. In case the CV between the samples was higher than 10%, the sample was excluded from analysis.

### Statistical analysis

The baseline characteristics gender and HLA risk group were compared using a Chi-square test. For analysis of I-FBAP concentration prior to seroconversion, all samples taken in the nonCD group and in the CD group were stratified into age categories: 0-3 months of age at sampling; 4-6 months; 7-9 months; 10 - 12 months; 13-24 months and >24 months. I-FABP concentration in each age category was compared between the CD and nonCD group using Mann Whitney U test.

The comparisons were repeated in the subgroup of children who developed CD at a young age, before 3 years old, compared to nonCD controls.

For comparison of I-FABP development over time between the CD and nonCD group, a linear mixed model analysis was conducted including group (CD and nonCD), age in months, and their interaction as fixed variables in the model. Since the age effect on I-FABP might be non-linear, we also considered a quadratic and cubic age trend, including their interactions with group. As for the random part, a random intercept on child level only model as well as two random intercept and slope models, assuming a variance components or unstructured covariance structure, were considered. A top-down procedure was then used to determine the final model.

Hereafter, a post-hoc analysis was performed in the subgroup of children who developed CD at a young age (before 3 years old) compared to nonCD controls. Here, the same linear mixed model with top-down procedure was used. IBM SPSS Statistics for Windows version 28.0 was used for all analyses, where two-sided p-values  $\leq 0.05$  were considered statistically significant.

### 3. Results

#### *Baseline characteristics of subjects*

I-FABP concentration was measured in 488 samples from 137 children, 61 in the CD group and 76 in the nonCD group (see table 1). Out of the 61 children with CD, 27 developed the disease within the first three years of life and the first case was diagnosed at 13 months of age. All measured samples could be used for analysis, as no VC between the duplicates exceeded 10%. There was no significant difference in gender between the CD and nonCD group ( $p=0,75$ ) and no significant difference in the distribution of HLA risk groups between the CD and the nonCD group ( $p=0,37$ ).

**Table 1:** Baseline characteristics of study subjects, children who developed celiac disease (CD) (CD group) and children who did not develop CD (nonCD group). Frequencies were compared Using a chi-square test.

	CD group (n= 61)	NonCD group (n= 76)	Subgroup: CD group, CD diagnosis before 3 years age (n= 27)
<b>Female, no. (%)</b>	33 (54%)	43 (57%)	16 (59%)
<b>HLA risk group</b>			
1 14 (23%)	10 (13%)	5 (19%)	
2 8 (13%)	11 (14%)	3 (11%)	
3 27 (44%)	31 (41%)	14 (52%)	
4 12 (20%)	24 (32%)	5 (19%)	
<b>Age of seroconversion, range in months</b>	13-44 months	-	13-36 months

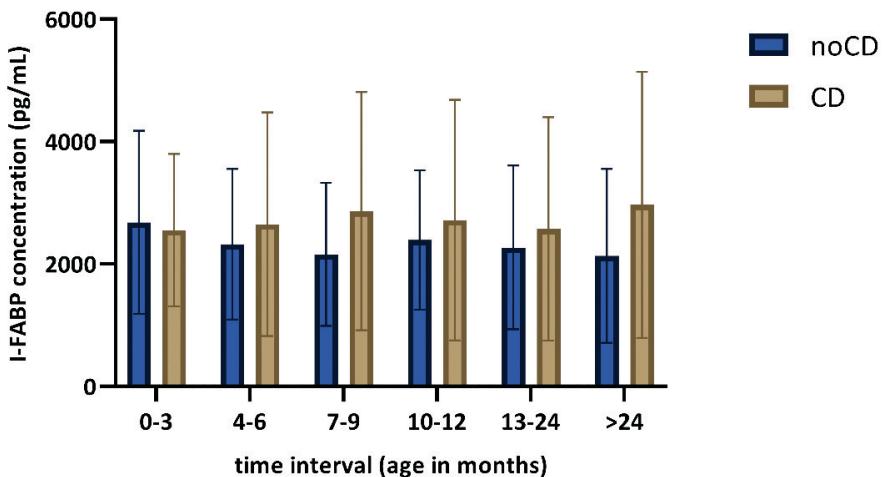
HLA risk groups were defined as: group 1: DQ2.5/DQ2.5 and DQ2.5/DQ2.2; group 2 DQ2.2/DQ7; group 3 DQ2.5/DQ7, DQ2.5/DQ8, and DQ2.5/other; group 4 DQ2.2/DQ2.2, DQ2.2/DQ8, and DQ8/DQ8; DQ2.2/other, DQ8/DQ7, and DQ8/other

#### *I-FABP concentration in first year of life prior to disease onset in different age categories*

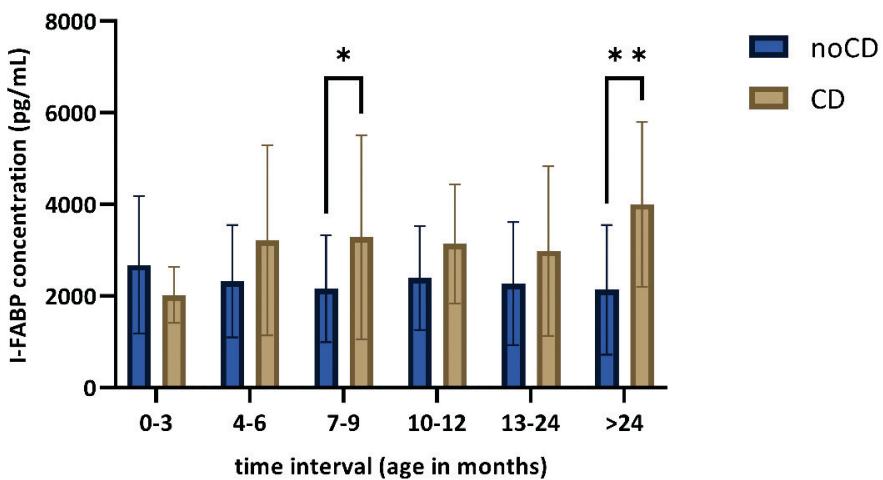
In the comparison of I-FABP concentration between the CD and nonCD group, samples were pooled into age categories (see figure 1). Only samples taken prior to seroconversion were included in this analysis. In the total group, no significant difference in I-FABP was found in any age category. The numerical difference in I-FABP between the CD and nonCD group at age 7-9 months was not statistically significant ( $p= 0,067$ ) (see fig 1A). In the samples taken in this age category ( 7- 9 months age), I-FABP concentration was significantly higher in the children who developed CD before the age of 3 years compared to the nonCD group ( $p=0,019$ ). There was also a significant difference at samples taken at 24 months and above ( $p=0,003$ ) (see fig 1B).

When comparing the I-FABP concentration between the CD and nonCD group in the other age categories, no significant differences were found.

A.



B.



**Figure 1.** I-FABP concentration in ascending age categories in children who develop celiac disease (CD group) and children who didn't develop CD (nonCD group) **A.** the entire group and **B.** subgroup of children that developed CD before 3 years age. Only including samples taken prior to seroconversion.

*Linear mixed model analysis of I-FABP development over time*

In the total group, no significant difference was found in pattern of I-FABP development over time using linear mixed model analysis.

A post-hoc analysis was performed on a subgroup, namely children who developed CD at a young age (before 3 years of age). Here, the interaction terms (group (CD;nonCD) and age) were not significant, but a statistically significant difference between the CD and nonCD group was found for I-FABP concentration after correction for age (irrespective whether a linear, quadratic or cubic trend was assumed, p values for group were 0.007, 0.006, 0.007, respectively).

#### **4. Discussion**

This study showed a marked difference in the course of I-FABP concentration over time in the first year of life in a subgroup of children who developed CD before turning three years old, compared to controls who didn't develop CD, in a cohort of European children with high disease risk. I-FABP is a highly sensitive and specific biomarker for small intestinal damage, as it is released into the bloodstream rapidly following damage to the enterocytes (16, 17). I-FABP has a short half-life in the bloodstream, which means that it can be used to detect acute and ongoing damage to the small intestine. Previous studies have shown that it is a reliable marker for intestinal damage in active CD and during recovery after initiation of a gluten free diet (GFD) (16, 18-20).

In the current study, in infants who develop CD in childhood, a difference was observed in the concentrations of this marker for small intestinal damage within the first year of life. This is compared to the nonCD group, also at risk to develop CD. It is possible to interpret this difference as a first sign of imminent CD development with disease onset already initiated, yet before seroconversion has occurred. Alternatively, it can be viewed as a risk factor for disease development. There is evidence suggesting that the latter may be the case. It is thus hypothesized that presence of intestinal damage, impairing barrier function, forms a possible risk factor for CD development (21, 22). However, this hypothesis is mainly built on research data focusing principally on barrier function, not enterocyte damage.

For instance, altered intestinal permeability has been reported in healthy first degree relatives of patients with CD (23). Abnormal intestinal permeability prior to disease onset has been observed in CD animal models as well (9). Further, functional pathway analysis of large GWAS data has shown an enrichment in four genes involved in intestinal barrier function, although their exact role is poorly characterized (21). In a study by Monsuur *et al.* risk to develop CD was 2.3-times

higher in individuals with a gene mutation that plays a role in actin remodeling of epithelial enterocytes, providing further evidence for the link between intrinsic barrier defects and CD risk (24). Taking this evidence into account, several researchers suggest barrier aberrations, whether intrinsic or induced by environmental factors such as infections and gut microbiota, form a necessary link in disease pathogenesis.

While our understanding of the immune system's altered response to gluten, specifically gliadin peptides, continues to improve, the mechanisms by which large amounts of gliadin can cross the barrier before disease onset and trigger this modified immune response remain unclear. Enterocyte damage and subsequent barrier alterations could facilitate this migration of gliadin across the barrier. To our knowledge, this is the first study in children at risk to develop CD, investigating barrier dysfunction by focusing specifically on enterocyte damage.

The age at which a difference in enterocyte damage was observed is notable. In samples taken at ages 2-3 months and 4-6 months, no significant difference between I-FABP concentration was found between the two groups, whereas a prominent difference was present in the second half of the first year of life. This concerns an interesting time window which follows an array of changes affecting the intestine and immune system of developing infants. Introduction of solid foods in general and gluten occur from 4-6 months onward, often accompanied by cessation or decreasing of breastfeeding or formula feeding and maturation of the gut microbiota composition (25). The difference in I-FABP concentration could be triggered by these factors, such as gluten introduction. Gliadin, for instance has been shown to have a direct effect on tight junction integrity of the small intestinal epithelium (26-28). And it has been suggested that differences in gluten intake may itself be a risk factor to develop CD (11, 29, 30). On the other hand, the altered progression in I-FABP concentration in children who develop CD could also be an effect of time and an intrinsic altered maturation of the epithelium compared to children who do not develop CD.

#### *Strength and weaknesses*

To our knowledge, this is the first study focusing on intestinal barrier damage in children at risk to develop CD in early life. The long follow-up period of the PreventCD study project provides a unique opportunity to compare children who do and do not develop CD, not only immediately before seroconversion, but from very early on.

The development of I-FABP concentration over time very early in life in healthy not at risk subjects is however largely unknown. Therefore, the relatively high I-FABP concentrations observed after birth in this cohort, in both the CD and

nonCD group need to be contextualized. It is unknown, whether the development of I-FABP concentrations in the nonCD group represent a normal physiological finding or whether the observations in this high-risk cohort already represent an overall altered level of I-FABP concentration early in life as compared to children with a low risk to develop CD. Comparing I-FABP values in the first year of life between this cohort to a group of low-risk infants would show whether high I-FABP concentrations after birth that decrease over time are a sign of a physiological process of gut maturation, or whether this finding is a result of an intrinsic altered barrier, as a risk factor for disease susceptibility in these high risk individuals. This next step could make the findings of this study even more striking, as we already observe differences in I-FABP patterns within the at-risk group. A further distinction between the entire group of at-risk children with low-risk children would be a novel finding that could contribute to our pathophysiological understanding of CD and its intrinsic versus environmental risk factors.

When interpreting the results, it must further be noted, that I-FABP is a biomarker to estimate intestinal barrier damage, enterocyte damage in particular. This study did not include an evaluation of barrier function in its entirety. The intriguing results of this study indicate that especially the first year of life should be of particular interest herein and future research should include other methods of assessing barrier integrity and functionality together in this period.

## **5. Future perspectives**

Intestinal barrier alterations early in life could be either indicators of or risk factors for CD development during childhood in individuals with a high risk to develop CD based on genetic susceptibility and family history. The results of this study warrant a more in depth investigation of gut maturation, functionality and damage early in life, and its relationship to CD development as well as interplay/causality with other risk factors for CD development. For this, tools should be combined, investigating the small intestinal barrier focusing on different aspects of functionality and structure.

Moreover, a comparison of children who develop CD with those who don't and those with a low or negligible risk to develop CD will yield additional insight into causality and pathophysiology of CD onset. In this, other risk factors to develop CD as well as barrier function should be taken into account.

Understanding the role of intestinal barrier damage in the pathogenesis of CD may add an integral puzzle piece in our understanding of disease development and the interplay between its risk factors. This could yield information on potential targets for disease prevention.

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# MONOSACCHARIDES AND GLIADIN INCREASE INTESTINAL BARRIER PERMEABILITY IN AN IN VITRO CELL MODEL

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## **Abstract**

### **Background**

Celiac disease (CD) is a common chronic disease whose disease etiology is not fully understood, but disruption of the intestinal barrier is hypothesized to play a pivotal role. Gluten consumption early in life and a “Westernized diet” are associated with an increased risk to develop CD in genetically predisposed individuals. The involved dietary components and their mechanism of action herein are unknown. In this study, we aimed to evaluate the effect of monosaccharides and gliadin on intestinal barrier function using a well-established *in vitro* model.

### **Methods**

A Caco-2 cell model was used, culturing enterocytes in a monolayer with distinct polarity. The luminal side was exposed to glucose, fructose and gliadin separately and in combination for one hour. Gliadin was digested *in vitro* with pepsin, trypsin, and chymotrypsin (GPTC) to mimic physiological conditions and diluted in culture medium in the desired concentrations for exposure experiments. Intestinal barrier function was evaluated by measuring Trans epithelial electrical resistance (TEER). Paracellular permeability was measured by assessing the flux of Fluorescein isothiocyanate (FITC) labeled 4-kDa dextran (FITC-4D) across the barrier. Results are based on triplicate experiments, each performed at least in duplicate.

### **Results**

TEER levels showed a mean decrease of 38% after exposure to 1 mg/mL GPTC as compared to control ( $p<0,0001$ ). Exposure to glucose and fructose (40  $\mu$ g/ml each) also led to a significant decrease in TEER (46,2 % and 26,9%, respectively;  $p<0,0001$  and  $p=0,0005$  respectively), but not at the lower concentration of 20  $\mu$ g/mL. FITC-4D flux was found to be increased after exposure to 20 and 40  $\mu$ g/ mL glucose ( $p=0,0199$  and  $p<0,0001$ , respectively), but not fructose. The effects of glucose and fructose were attenuated after combined exposure with 0,5 mg/ mL GPTC.

### **Discussion and conclusion**

This *in vitro* study showed that exposure of intestinal epithelial cells to glucose, fructose and digested gliadin dose-dependently increased intestinal permeability. This effect was attenuated after combined exposure of digested gliadin with the monosaccharides. Further investigation is needed to better understand the underlying mechanisms and potential interactions between monosaccharides and gliadin derivatives.

## 1. Introduction

Celiac disease (CD) is a chronic disease in which gluten triggers an auto-immune reaction resulting, among other down-stream effects, in damage to the small intestinal wall, characterized by an immune reaction and villous atrophy (1, 2). Gluten exposure and genetic susceptibility are risk factors that have to be present for CD to develop. However, only a fraction of genetically susceptible individuals develop CD, while the role of additional etiological factors are not fully understood. High amounts of gluten consumption early in life, as well as the “Westernized diet” have been reported to increase the risk to develop CD in genetically predisposed individuals (3-5). The exact dietary components involved, as well as their mechanism of action are, however, unknown. Common characteristics of the Westernized diet include high intake of calorie-dense products, refined carbohydrates, highly processed foods, and high intake of salt and saturated fats and low intake of fibers (6). In the generation R birth cohort study, a dietary pattern with low consumption of refined cereals and sweet beverages was associated with a lower risk to develop CD (7).

Currently, evidence is emerging for involvement of an impaired intestinal barrier function in CD onset (8, 9). It is hypothesized that increased permeability of the intestine facilitates large amounts of gliadin peptides to cross the barrier and encounter immune cells. This results in an increased exposure of gliadin or its derivatives to immune cells via antigen presenting cells, which will increase the risk of CD onset (5). The intestinal mucosal barrier is formed by the epithelial monolayer, covered by the mucus layer and gut microbiota, and the mucosa-associated lymphoid tissue (9, 10). Enterocytes, specialized epithelial cells for absorption, form the main component of the epithelial monolayer. Previous studies showed that gliadin, the immunogenic component of gluten increased intestinal permeability and led to tight junction (TJ) alterations in cell models and a gluten sensitive mouse model (11, 12). Most of these studies used undigested gliadin or applied a pre-digestion with pepsin and trypsin only (11). Moreover, key constituents from a Western-style diet may also impact intestinal barrier function. High fat intake and some emulsifiers for example have been shown to be associated with an increased intestinal permeability (13, 14). In contrast, vitamin D and fibers directly or indirectly via the microbial production of short chain fatty acids may strengthen the intestinal barrier (11, 15-19). The impact of refined carbohydrates, alone or in combination with gliadin, are less well studied.

In this study, we aimed to evaluate the effect of exposure to high dosages of monosaccharides alone and in combination with gliadin on intestinal barrier function using a well-established *in vitro* Caco-2 cell culture model. We hypothesized that the monosaccharides alone would lead to an increase in intestinal permeability and that the combination of a monosaccharide with gliadin may lead to an additive or even synergistic increase in permeability.

## **2. Material and Methods**

### *Cell line and culture conditions*

Caco-2 cells (ATCC Rockville, MD) were grown in T75 flasks using Dulbecco's Modified Eagle Medium (DMEM; Lonza Benelux BV) culture medium containing 4,5 g/l glucose and L-glutamine, as described previously (20). First, cells were cultured in flasks until they were 80-90% confluent. Then, Caco-2 cells were seeded at a density of 100.000 cells/well and grown for 21 days on a collagen-coated polycarbonate membrane in a trans-well system (0,33 cm<sup>2</sup>, 0,4µm pore size) into monolayers (Costar, Cambridge, MA). Caco-2 cells were cultured at 37°C in a 95% air/5% CO<sub>2</sub> atmosphere.

### *Test compounds*

To mimic intestinal digestion of gliadin, gliadin from wheat (G3375, Sigma-Aldrich, St Louis, USA) was digested with pepsin/trypsin (PT-gliadin) or pepsin/trypsin/ chymotrypsin (PTC-gliadin) as described previously (21). In short, gliadin was dissolved in 5% formic acid with a final concentration of 1 mg/ml and then incubated with 0,01 mg/ml pepsin (P6887, Sigma-Aldrich) with continuous shaking for 2 hours at 37°C to mimic gastric digestion. After lyophilisation, samples were dissolved in 100mM ammonium bicarbonate. Then 1 mg/ml trypsin (T9201, Sigma-Aldrich) dissolved in hydrogen chloride (1 mM HCl), was added to the samples (0,01 mg/ml) for 4 hours at 37°C, with continuous shaking. For the PTC-gliadin, 0,01 ml/ml chymotrypsin was added together with the trypsin (C7762, Sigma-Aldrich). After incubation, the samples were boiled for 5 minutes at 100°C. After another lyophilisation, the samples were dissolved in ammonium bicarbonate 100mM and stored at 4°C. To confirm digestion of gliadin into PT-gliadin and PTC-gliadin an SDS-page gel electrophoresis was performed (1 mg/ml and maximum concentration). Mini-protean TGX 20% gel was used (Biorad, Hercules, USA) and electrophoresis was started at 60 V for 5 minutes and increased to 120 V for 65 minutes. After electrophoresis, the gel was washed and incubated with coomassie blue overnight.

Concentrations of PT-gliadin and PTC-gliadin were determined using a BCA protein assay kit (ThermoFisher Scientific, Waltham, USA). PT-gliadin and PTC-gliadin was diluted in culture medium in the desired concentrations for cell culture experiments.

Glucose and fructose (G7021 and F0127, respectively, Sigma-Aldrich, St Louis, USA), mimicking a high intake of refined carbohydrates, were dissolved in 1x PBS at a final concentration of 500 mg/ml and stored at 4°C until use. For the experiments, the samples were diluted in culture medium in the desired concentrations. Culture medium only was used as negative control and hydrogen peroxide 100mM as a positive control.

#### *Measurement of transepithelial electrical resistance and FITC-D4 permeation*

After monolayers had developed in the transwell inserts, they were exposed to test compounds for one hour to mimic small intestinal exposure. First, PT and PTC-gliadin were added in increasing concentrations from 0,25 mg/mL to 1 mg/mL. Next, glucose and fructose were added in increasing concentrations from 20 µg/mL to 40 µg/mL. Lastly, a combination of 0,5mg/mL PTC-gliadin and either glucose or fructose (20 µg/mL; 40 µg/mL) was studied.

Transepithelial electrical resistance (TEER) measurement was used to determine electrical resistance over the monolayers, as indicator for barrier function. TEER was measured at baseline and after one hour of incubation with the test compounds using an EVOM2 Epithelial Volthometer. TEER values were corrected by subtracting background resistance of a blank without cells and containing medium only.

3

After the TEER measurement, Fluorescein isothiocyanate (FITC) labeled 4-kDa dextran (1 mg/ml FITC-D4; Sigma- Amsterdam, the Netherlands) was added to the luminal side of the cells and incubated for one hour at 37°C. The flux of FITC-D4 over the membrane was assessed by measuring fluorescence in the luminal and basolateral medium compartment with a spectrophotometer at 485 nm excitation and 530 nm emission. The ratio between the luminal and basolateral FITC-D4 concentrations was calculated and corrected for values measured in a blank duplicate.

#### *Testing cell viability*

To test for cytotoxicity of test compounds, the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay for cell viability was performed (22). Caco-2 cells were incubated with PT-gliadin (1 mg/ml), glucose and fructose (40 µg/ml) or culture medium as a negative control for one hour. Hereafter, cells were incubated with Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma, M2128) for 2,5 hours at 37°C (ratio Thiazolyl Blue Tetrazolium Bromide: culture medium is 1:9). Then, cells were incubated with 10% sodium dodecyl sulfate (SDS) overnight at 37°C. Absorbance was measured at a wavelength of 570 nm and background absorbance was measured at a wavelength of 690 nm with a spectrophotometer. Experimental conditions were compared to the medium only as negative control.

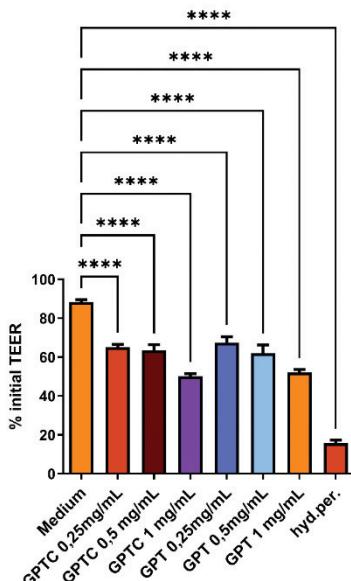
In addition, lactate dehydrogenase (LDH) activity was measured as marker for cell damage as described previously by Chan *et al.* (23). In short, after one hour exposure to the test compound, supernatant was collected from the trans wells and 9% Triton X-100 was added as emulsifier in a 96 well plate. LDH assay buffer was added to the supernatants and incubated for 30 minutes at room temperature, protected from light. After adding the stop solution (1M acetic acid), absorbance was measured with a spectrophotometer at a wavelength of 510 nm.

### Statistical analysis

All data analyses were conducted with GraphPad Prism software package (GraphPad Software, San Jose, CA). Data are expressed as means  $\pm$  SD of triplicate experiments, each performed at least in duplicate. For all outcome measures, the coefficient of variation (CV) was calculated between the duplicates and triplicates respectively. Only replicates with a CV  $\leq 10\%$  were included in the analysis. One-way ANOVA and Tukey's post hoc tests were performed to compare between TEER and FITC changes in the different experimental conditions, considering difference of  $p < 0,05$  as statistically significant. Kruskal-Wallis test was used to analyze results of the MTT and LDH viability assays. All p-values presented were corrected for multiple testing.

## 3. Results

To determine the effect of separate exposure of glucose and fructose and their combination on intestinal barrier function, we used a Caco-2 cell culture model and assessed electrical resistance and flux of FITC-D4 across the monolayer after one hour of exposure.



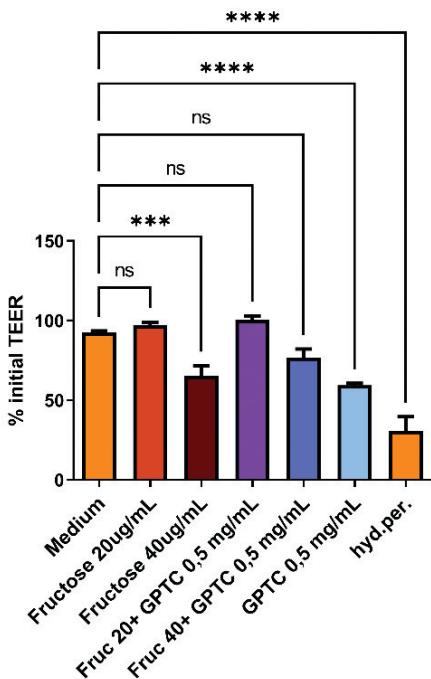
**Figure 1.** Change in TEER (in %) in Caco-2 cell monolayers after exposure to digested gliadin in different concentrations for one hour. Gliadin was pre-digested with either pepsin and trypsin (GPT), or with pepsin trypsin and chymotrypsin (GPTC). Conditions were compared versus medium control using ANOVA with Tukey's post-hoc test correcting for multiple testing.. Significant differences are indicated with \*, depending on level of p-value: \*: $p < 0,05$ ; \*\*: $p < 0,01$ ; \*\*: $p < 0,001$  \*\*\*\*:  $p < 0,0001$ .

Abbreviations: TEER: Transepithelial electrical resistance; GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; GPT: Gliadin digested with pepsin and trypsin; hyd.per: Hydrogen peroxide

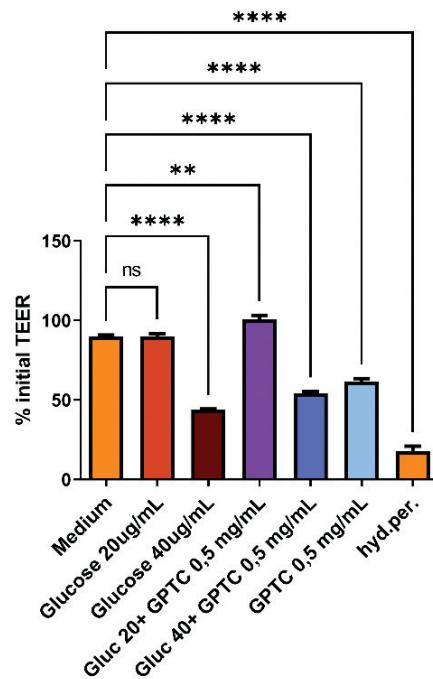
We first compared the effect of gliadin pre-digested with pepsin and trypsin only (GPT), or after pre-digestion with pepsin, trypsin and chymotrypsin (GPTC), and found a significant dose-dependent decrease in TEER values after exposure to both GPT and GPTC as compared to the medium control (see fig 1 and supplementary table 1; all  $p < 0.001$ ). Exposure to 1 mg/mL GPTC and GPT resulted in an average reduction of TEER of 38,2% and 36,2%, respectively, compared to control (both  $p < 0.0001$ ; see also supplemental table 1 for pairwise comparisons between conditions). All  $p$  values were corrected for multiple testing.

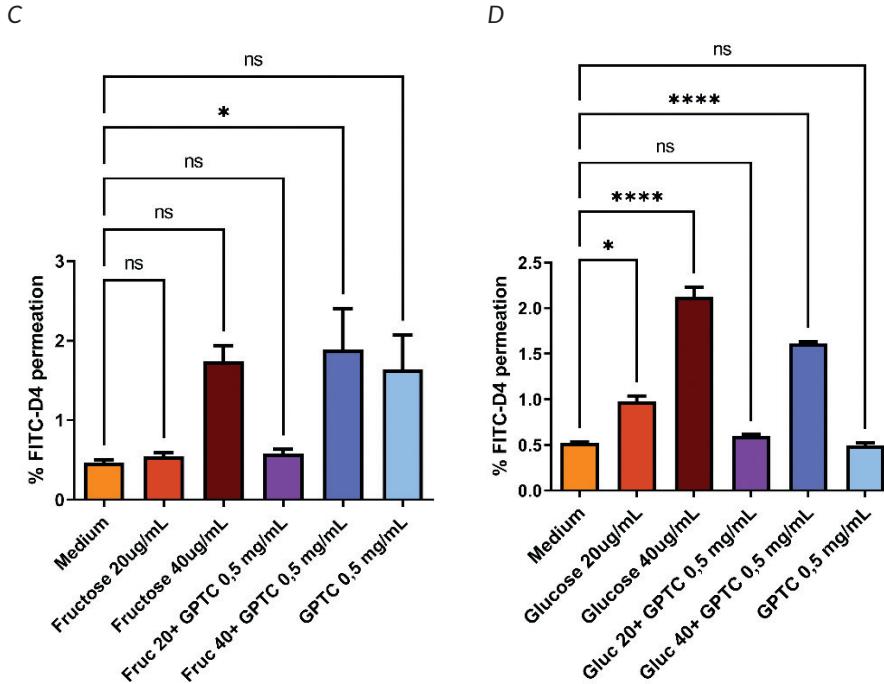
Further experiments on the effect of glucose and fructose alone and in combination with gliadin were performed with GPTC to best mimic the *in vivo* setting and using a concentration of 0,5 mg/mL to study potential additive or synergistic effects when combined with glucose or fructose.

A



B





**Figure 2.** TEER (2A&B) and FITC permeation (2C&D) in percent after one hour of exposure to 20  $\mu$ g/mL and 40  $\mu$ g/mL fructose and glucose; alone and in combination with 0,5 mg/mL GPTC. Conditions were compared using ANOVA with Tukey's post-hoc test correcting for multiple testing. Only comparisons versus medium control are shown, for other pairwise comparisons see supplementary tables 2-5. Significant differences are indicated with \*, depending on level of p-value: \*:p<0,05; \*\*:p<0,01; \*\*\*:p<0,001 \*\*\*\*: p<0,0001

Abbreviations: TEER: Transepithelial electrical resistance; GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; hyd.per: Hydrogen peroxide

#### TEER values:

Exposure of the Caco-2 monolayers to 40  $\mu$ g/mL glucose or fructose showed a significant reduction in TEER values ( $p<0,0001$  and  $p=0,0005$ , respectively) (see fig 2A, 2B and supplementary tables 2, 3). The mean reduction versus medium control appeared to be more pronounced for 40  $\mu$ g/mL glucose as compared to fructose (46,2% vs 26,9%). No significant effect was observed after exposure to the lower concentration of 20  $\mu$ g/mL. Exposure to 0,5 mg/mL GPTC alone also resulted in a significant decrease of TEER in all experiments, with a mean reduction in TEER of 28% ( $p<0,0001$ ) (see fig 1, 2A, 2B). The combined exposure of 0,5 mg/ mL GPTC with 20  $\mu$ g/mL fructose or glucose did not result in a significant decrease in TEER as compared to medium control. Even a slight but significant increase (10,6%;  $p=0,0031$ ) in TEER compared to medium control was observed in Caco-2 monolayers when exposed to glucose and GPTC in combination (see fig 2B).

The combination of 40 µg/mL glucose and 0,5 mg/mL GPTC resulted in a significant reduction of TEER by 35,5% compared to the medium control ( $p<0,0001$ ) (see fig 2B). The combination of 40 µg/mL fructose and 0,5 mg/mL GPTC had no significant effect on TEER when compared to the medium control ( $p=0,1345$ ). In contrast, exposure to both compounds alone induced a significant reductions in TEER (see fig 2A).

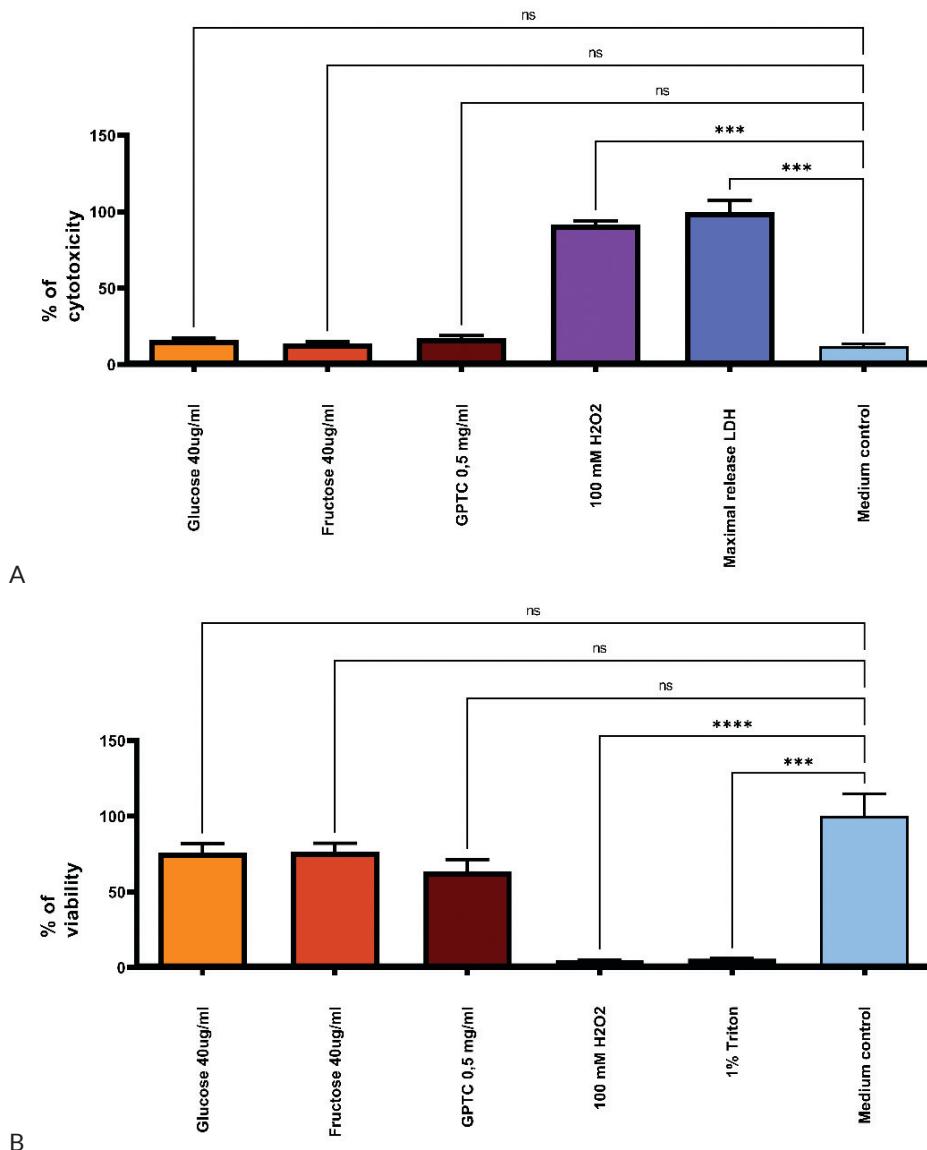
*FITC-D4 permeation:*

In line with the TEER results, exposure to the glucose also resulted in a significant increase in FITC permeation across the monolayer (see fig 2D). The flux of FITC-D4 was significantly and dose-dependently increased after exposure to 20 µg/mL glucose ( $p=0,0199$ ) and 40 µg/mL glucose ( $p<0,0001$ ) compared to the medium control (see fig 2D). The effect was attenuated in combination with GPTC, which showed no significant difference in FITC-D4 permeation after exposure to 20 µg/mL glucose when combined with 0,5 mg/mL GPTC. The increase in FITC-D4 after exposure to 40 µg/mL glucose remained significant even in combination with 0,5 mg/mL GPTC when compared to medium control ( $p<0,0001$ ). However, this effect was significantly weaker as compared to exposure with 40 µg/mL glucose alone ( $p=0,0060$ ; see also supplemental table 5 for pairwise comparisons between conditions).

Exposure to fructose in both concentrations did not lead to a statistically significant increase in FITC-D4 flux compared to the medium control. Although an increased FITC-D4 was observed after exposure to fructose 40 µg/ml, this was not significant ( $p=0,057$ ). The combination of 0,5 mg/mL GPTC with fructose 40 µg/mL, but not 20 µg/ml, led to a significant increase in FITC-D4 permeation ( $p<0,0001$  and  $p = 0,0331$ , respectively) (see fig 2 C,D and supplementary table 4).

*Cell toxicity:*

In order to assess whether the exposure of the Caco-2 cells to the test compounds lead to cell death, an MTT and LDH cell viability assay were performed (see fig 3). There was no significant difference in LDH activity between the medium control condition and exposure to 40 µg/mL glucose, fructose or 0,5 mg/mL GPTC (see figure 3A). Similarly, there was no difference in cell viability using the MTT assay between the control condition and the exposure conditions (see figure 3B).



**Figure 3.** LDH (A) and MTT (B) viability assay of Caco-2 cells exposed to different stressors for one hour. Conditions were compared using Kruskal Wallis test. The comparisons with medium control are shown. Significant differences are indicated with \*, depending on level of p-value: \*:p<0,05; \*\*:p<0,01; \*\*\*:p<0,001 \*\*\*\*: p<0,0001  
 Abbreviations: GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin

## **4. Discussion**

We evaluated the effect of exposure to monosaccharides with and without gliadin on intestinal barrier function using a well-established *in vitro* cell culture model. The exposure to high dosages glucose and fructose and *in vitro* digested gliadin led to decreased TEER levels and increased permeation of FITC-D4. Remarkably, the combination of either fructose or glucose with gliadin attenuated the effect of all separate compounds. The exposure of the Caco-2 cells to both 20 and 40  $\mu$ g/ml concentrations of glucose but not fructose also led to increased paracellular flux of the large molecule FITC-D4, indicating barrier disruption in line with the TEER results. This was also in part diminished after co-exposure with gliadin. No effect was shown on cell viability by neither of the compounds tested.

Gliadin, the immunogenic component of gluten triggers the autoimmune reaction in CD at the site of the basolateral membrane of the small intestine (24). A Western diet, as characterized by an increased intake of high fructose corn syrup and glucose, has been hypothesized to be a potential risk factors for CD development (5). Moreover, a dietary pattern characterized by low consumption of sugar has been proposed to have a protective effect on CD risk (25). We postulate that the effect of monosaccharides on intestinal barrier permeability is contributing factor to the increased CD risk through sugar intake and may facilitate gliadin passage across the barrier, enabling increased contact of gliadin with the gut associated immune system. Therefore, we studied the effect of fructose and glucose alone and in combination with gliadin on intestinal barrier function.

We showed that exposure of relative high, yet physiological, dosages of glucose and fructose (40  $\mu$ g/mL) to the intestinal epithelium resulted in a strong increase in permeability, as supported by significant decreases in TEER and increases in FITC-D4 permeation. Also a significant impaired barrier function was found after exposure to 20  $\mu$ g/mL glucose, but not fructose. Moreover, after exposure to glucose, the flux of the relatively large FITC-D4 molecule across the barrier was increased, indicating an amplified paracellular flux due to exposure to glucose. Interestingly this effect was not seen after exposure to fructose. Combined effects were dose-dependent and more pronounced after glucose as compared to fructose. The different effects of glucose and fructose on the barrier can relate to the ions involved in their transport across the membrane. Fructose enters the enterocyte through glucose transporter 5 (GLUT5/SLC2A5), a specific fructose transporter located on the apical enterocyte membrane (26, 27). Notably, unlike glucose transport, this mechanism operates independently of sodium uptake. Glucose absorption occurs via the active transporter sodium-glucose co-transporter 1 (SGLT1) that also functions as a passive water channel (28). This process is driven by a sodium gradient. which is generated by  $\text{Na}^+ \text{-K}^+$ -ATPase located on the

basolateral membrane of the enterocytes (29, 30). The ion flux, particularly of sodium, associated with glucose transport, could alter permeability through its influence on TJs. Furthermore, altered ion fluxes can also influence transepithelial resistance without affecting paracellular permeability.

Gliadin, the immunogenic component of gluten, known to trigger the autoimmune reaction in CD, has also been identified as a potential risk factor for CD development itself; *i.e.* ingestion of large amounts of gluten early in life has been associated with an increased risk to develop CD (3, 4).

One of the proposed mechanisms of action herein is the increased intestinal permeability caused by gliadin itself. This allows a greater amount of gliadin to pass the intestinal barrier, resulting in an increased contact with immune cells via antigen presenting cells through HLA encoded MHC II complexes (DQ2/8) and the subsequent CD-specific aberrant immune reaction. In this study, we observed a significant decrease in TEER after exposure to GPTC, indicating a strongly increased permeability.

In line with our findings, Drago *et al.* showed a marked increase of intestinal barrier permeability after exposure to PT digested gliadin (1 mg/ml) for 90 and 180 minutes in a Caco-2 cell model. They further showed that this may be a result of gliadin-induced release of zonulin and rearrangement of the cytoskeleton, as well as redistribution of TJ components such as zonula occludens-1 (ZO-1) (31). In our study, we chose a shorter exposure time of 60 minutes and chymotrypsin was added to the digestion process of gliadin before exposure to closer mimic the *in vivo* situation. Enzymatic degradation analysis has suggested that trypsin and chymotrypsin degrade different epitopes of gliadin during human digestion (32). Chymotrypsin digestion appears to yield more immunogenic epitopes identified in CD pathophysiology, which can bind to HLA-DQ receptors of antigen presenting cells and leading to T cell activation (32).

When making the translation to dietary intake and dietary patterns, the combined effect of sugars and gliadin together are of particular interest. Remarkably, the combined exposure of glucose or fructose with gliadin did not enlarge the reduction in TEER compared to gliadin exposure alone. We did not observe an additive or synergistic effect, in fact, overall the combination of glucose and fructose with GPTC even attenuated the effects of all compounds alone. This suggests that the presence of monosaccharides can have a dual effect depending on the matrix. However, it is important to note that the effect of the combined exposure was not completely protective, as a significant increase in FITC-D4 permeation and a significant decrease in TEER was still observed when high concentrations of glucose or fructose were combined with GPTC.

The different effects observed in this study when examining dietary components alone and in combination with each other show the limitations of cell models in translation of effects to the physiological condition in humans. When investigating the effect of dietary intake on intestinal permeability, combination of dietary components resembling true dietary intake is important. This warrants further studies on intestinal permeability in human subjects. This further enables a true representation of complex meals also including emulsifiers and other food additives, the impact of food processing (*i.e.* heating), as well as other involved factors such as the gut microbiota. When studying the link with CD risk, such human studies could compare the effect of diet on intestinal permeability in at-risk individuals to low-risk controls using for example a multi-sugar test (33-36).

Further investigation is needed to better understand the underlying mechanisms and potential interactions between gliadin and monosaccharides, taking into account different concentrations. A first step could be to examine differences in gene expression between the exposure conditions in order to identify the involved cellular pathways.

Some limitations of this study are important to take into account. Although performed an *in vitro* digestion of gliadin, it should be noted that this will not completely resemble the *in vivo* situation. Further, it should be noted that relative changes in FITC permeation were rather small. Although the direct comparison between conditions is still valid, we recently noted that this was due to a technical limitation of the inserts used. However, our overall conclusions are substantiated by robust results from TEER analyses based on at least triplicate experiments with all conditions tested at least in duplicate. Finally, it should be noted that Caco-2 is an immortalized human colorectal adenocarcinoma cell line, which not fully captures the physiological situation and exhibit stronger tight junctions (37). We chose our exposure to reflect small intestinal exposure. Future host-derived organoid models better reflect the different cell types present as well as enable incorporation of host genetics.

Overall, our findings suggest that both digested gliadin and monosaccharides, particularly at high concentrations, can compromise the integrity and function of the intestinal barrier. These results contribute to our understanding of the potential mechanisms underlying the increased risk of CD associated with high gluten consumption and Western-style diets. Further research is needed to elucidate the specific molecular pathways involved as well as to further investigate the impact of different combination and the overall diet. Understanding the role of dietary components in intestinal barrier dysfunction could potentially inform preventive and therapeutic strategies for CD and other related disorders.

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## Supplementary Material

**Supplementary table 1:** Adjusted p-values of Tukey's post hoc test comparing difference in TEER in Caco-2 cell monolayer after exposure to digested gliadin in different concentrations for one hour. Gliadin was pre-digested with either pepsin and trypsin (GPT), or with pepsin trypsin and chymotrypsin (GPTC). For graphical representation see figure 1.

<i>Tukey's multiple comparisons test</i>	Mean Difference	95% confidence interval of difference	Adjusted P Value
Medium vs. GPTC 0,25mg/mL	23,17	12,17 to 34,17	<0,0001
Medium vs. GPTC 0,5 mg/mL	24,94	13,94 to 35,94	<0,0001
Medium vs. GPTC 1 mg/mL	38,19	27,19 to 49,19	<0,0001
Medium vs. GPT 0,25mg/mL	21,06	10,06 to 32,06	<0,0001
Medium vs. GPT 0,5mg/mL	26,47	15,47 to 37,47	<0,0001
Medium vs. GPT 1 mg/mL	36,20	25,20 to 47,20	<0,0001
Medium vs. hyd.per.	72,74	60,44 to 85,04	<0,0001
GPTC 0,25mg/mL vs. GPTC 0,5 mg/mL	1,766	-9,234 to 12,77	0,9995
GPTC 0,25mg/mL vs. GPTC 1 mg/mL	15,02	4,022 to 26,02	0,0021
GPTC 0,25mg/mL vs. GPT 0,25mg/mL	-2,109	-13,11 to 8,890	0,9985
GPTC 0,25mg/mL vs. GPT 0,5mg/mL	3,297	-7,703 to 14,30	0,9773
GPTC 0,25mg/mL vs. GPT 1 mg/mL	13,03	2,029 to 24,03	0,0109
GPTC 0,25mg/mL vs. hyd.per.	49,57	37,27 to 61,86	<0,0001
GPTC 0,5 mg/mL vs. GPTC 1 mg/mL	13,26	2,256 to 24,26	0,0091
GPTC 0,5 mg/mL vs. GPT 0,25mg/mL	-3,875	-14,87 to 7,125	0,9461
GPTC 0,5 mg/mL vs. GPT 0,5mg/mL	1,531	-9,468 to 12,53	0,9998
GPTC 0,5 mg/mL vs. GPT 1 mg/mL	11,26	0,2636 to 22,26	0,0415
GPTC 0,5 mg/mL vs. hyd.per.	47,80	35,50 to 60,10	<0,0001
GPTC 1 mg/mL vs. GPT 0,25mg/mL	-17,13	-28,13 to -6,131	0,0003
GPTC 1 mg/mL vs. GPT 0,5mg/mL	-11,72	-22,72 to -0,7251	0,0297
GPTC 1 mg/mL vs. GPT 1 mg/mL	-1,993	-12,99 to 9,007	0,9989
GPTC 1 mg/mL vs. hyd.per.	34,55	22,25 to 46,84	<0,0001
GPT 0,25mg/mL vs. GPT 0,5mg/mL	5,406	-5,594 to 16,41	0,7614
GPT 0,25mg/mL vs. GPT 1 mg/mL	15,14	4,138 to 26,14	0,0019
GPT 0,25mg/mL vs. hyd.per.	51,68	39,38 to 63,97	<0,0001
GPT 0,5mg/mL vs. GPT 1 mg/mL	9,732	-1,267 to 20,73	0,1159
GPT 0,5mg/mL vs. hyd.per.	46,27	33,97 to 58,57	<0,0001
GPT 1 mg/mL vs. hyd.per.	36,54	24,24 to 48,84	<0,0001

Abbreviations: TEER: Transepithelial electrical resistance; GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; GPT: Gliadin digested with pepsin and trypsin; hyd.per: Hydrogen peroxide

**Supplementary table 2:** Adjusted p-values of Tukey's post hoc test comparing difference in TEER in Caco-2 cell monolayer after exposure to fructose and digested gliadin in different concentrations for one hour. Gliadin was pre-digested with pepsin trypsin and chymotrypsin (GPTC). For graphical representation see figure 2A.

<b>Tukey's multiple comparisons test</b>	<b>Mean Difference</b>	<b>95% confidence interval of difference</b>	<b>Adjusted P Value</b>
Medium vs. Fructose 20ug/mL	-4,836	-22,20 to 12,53	0,9738
Medium vs. Fructose 40ug/mL	26,86	9,496 to 44,23	0,0005
Medium vs. Fruc 20+ GPTC 0,5 mg/mL	-7,900	-25,27 to 9,468	0,7820
Medium vs. Fruc 40+ GPTC 0,5 mg/mL	15,57	-2,644 to 33,79	0,1345
Medium vs. GPTC 0,5 mg/mL	33,10	15,73 to 50,47	<0,0001
Medium vs. hyd.per.	61,58	42,16 to 80,99	<0,0001
Fructose 20ug/mL vs. Fructose 40ug/mL	31,70	14,33 to 49,07	<0,0001
Fructose 20ug/mL vs. Fruc 20+ GPTC 0,5 mg/mL	-3,064	-20,43 to 14,30	0,9976
Fructose 20ug/mL vs. Fruc 40+ GPTC 0,5 mg/mL	20,41	2,192 to 38,62	0,0201
Fructose 20ug/mL vs. GPTC 0,5 mg/mL	37,93	20,57 to 55,30	<0,0001
Fructose 20ug/mL vs. hyd.per.	66,41	46,99 to 85,83	<0,0001
Fructose 40ug/mL vs. Fruc 20+ GPTC 0,5 mg/mL	-34,76	-52,13 to -17,40	<0,0001
Fructose 40ug/mL vs. Fruc 40+ GPTC 0,5 mg/mL	-11,29	-29,51 to 6,923	0,4651
Fructose 40ug/mL vs. GPTC 0,5 mg/mL	6,234	-11,13 to 23,60	0,9145
Fructose 40ug/mL vs. hyd.per.	34,71	15,29 to 54,13	<0,0001
Fruc 20+ GPTC 0,5 mg/mL vs. Fruc 40+ GPTC 0,5 mg/mL	23,47	5,256 to 41,69	0,0051
Fruc 20+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	41,00	23,63 to 58,37	<0,0001
Fruc 20+ GPTC 0,5 mg/mL vs. hyd.per.	69,48	50,06 to 88,89	<0,0001
Fruc 40+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	17,53	-0,6893 to 35,74	0,0656
Fruc 40+ GPTC 0,5 mg/mL vs. hyd.per.	46,00	25,82 to 66,18	<0,0001
GPTC 0,5 mg/mL vs. hyd.per.	28,48	9,059 to 47,90	0,0011

Abbreviations: TEER: Transepithelial electrical resistance; GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; GPT: Gliadin digested with pepsin and trypsin; hyd.per: Hydrogen peroxide

**Supplementary table 3:** Adjusted p-values of Tukey's post hoc test comparing difference in TEER in Caco-2 cell monolayer after exposure to glucose and digested gliadin in different concentrations for one hour. Gliadin was pre-digested with pepsin trypsin and chymotrypsin (GPTC). For graphical representation see figure 2B.

<b>Tukey's multiple comparisons test</b>	<b>Mean Difference</b>	<b>95% confidence interval of difference</b>	<b>Adjusted P Value</b>
Medium vs. Glucose 20ug/mL	-0,06197	-8,239 to 8,115	>0,9999
Medium vs. Glucose 40ug/mL	46,18	38,36 to 54,01	<0,0001
Medium vs. Gluc 20+ GPTC 0,5 mg/mL	-10,59	-18,42 to -2,764	0,0031
Medium vs. Gluc 40+ GPTC 0,5 mg/mL	35,54	27,71 to 43,37	<0,0001
Medium vs. GPTC 0,5 mg/mL	28,20	20,37 to 36,02	<0,0001
Medium vs. hyd.per.	72,06	62,62 to 81,50	<0,0001
Glucose 20ug/mL vs. Glucose 40ug/mL	46,25	38,42 to 54,07	<0,0001
Glucose 20ug/mL vs. Gluc 20+ GPTC 0,5 mg/mL	-10,53	-18,36 to -2,702	0,0033
Glucose 20ug/mL vs. Gluc 40+ GPTC 0,5 mg/mL	35,60	27,78 to 43,43	<0,0001
Glucose 20ug/mL vs. GPTC 0,5 mg/mL	28,26	20,43 to 36,09	<0,0001
Glucose 20ug/mL vs. hyd.per.	72,12	62,68 to 81,56	<0,0001
Glucose 40ug/mL vs. Gluc 20+ GPTC 0,5 mg/mL	-56,78	-64,24 to -49,31	<0,0001
Glucose 40ug/mL vs. Gluc 40+ GPTC 0,5 mg/mL	-10,64	-18,11 to -3,178	0,0017
Glucose 40ug/mL vs. GPTC 0,5 mg/mL	-17,99	-25,45 to -10,52	<0,0001
Glucose 40ug/mL vs. hyd.per.	25,87	16,73 to 35,01	<0,0001
Gluc 20+ GPTC 0,5 mg/mL vs. Gluc 40+ GPTC 0,5 mg/mL	46,13	38,67 to 53,60	<0,0001
Gluc 20+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	38,79	31,32 to 46,25	<0,0001
Gluc 20+ GPTC 0,5 mg/mL vs. hyd.per.	82,65	73,51 to 91,79	<0,0001
Gluc 40+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	-7,347	-14,81 to 0,1173	0,0560
Gluc 40+ GPTC 0,5 mg/mL vs. hyd.per.	36,52	27,37 to 45,66	<0,0001
GPTC 0,5 mg/mL vs. hyd.per.	43,86	34,72 to 53,00	<0,0001

Abbreviations: TEER: Transepithelial electrical resistance; GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; GPT: Gliadin digested with pepsin and trypsin; hyd.per: Hydrogen peroxide

**Supplementary table 4:** Adjusted p-values of Tukey's post hoc test comparing difference in FITC permeation in Caco-2 cell monolayer after exposure to fructose and digested gliadin in different concentrations for one hour. Gliadin was pre-digested with pepsin trypsin and chymotrypsin (GPTC). For graphical representation see figure 2C.

<i>Tukey's multiple comparisons test</i>	Mean Difference	95% confidence interval of difference	Adjusted P Value
Medium vs. Fructose 20ug/mL	-0,08268	-1,278 to 1,113	0,9998
Medium vs. Fructose 40ug/mL	-1,272	-2,582 to 0,03714	0,0574
Medium vs. Fruc 20+ GPTC 0,5 mg/mL	-0,1100	-1,305 to 1,085	0,9992
Medium vs. Fruc 40+ GPTC 0,5 mg/mL	-1,422	-2,731 to -0,1122	0,0331
Medium vs. GPTC 0,5 mg/mL	-1,172	-2,482 to 0,1373	0,0832
Fructose 20ug/mL vs. Fructose 40ug/mL	-1,190	-2,385 to 0,005729	0,0512
Fructose 20ug/mL vs. Fruc 20+ GPTC 0,5 mg/mL	-0,02735	-1,097 to 1,042	>0,9999
Fructose 20ug/mL vs. Fruc 40+ GPTC 0,5 mg/mL	-1,339	-2,534 to -0,1436	0,0282
Fructose 20ug/mL vs. GPTC 0,5 mg/mL	-1,089	-2,285 to 0,1059	0,0769
Fructose 40ug/mL vs. Fruc 20+ GPTC 0,5 mg/mL	1,162	-0,03308 to 2,358	0,0572
Fructose 40ug/mL vs. Fruc 40+ GPTC 0,5 mg/mL	-0,1494	-1,459 to 1,160	0,9977
Fructose 40ug/mL vs. GPTC 0,5 mg/mL	0,1002	-1,209 to 1,410	0,9997
Fruc 20+ GPTC 0,5 mg/mL vs. Fruc 40+ GPTC 0,5 mg/mL	-1,312	-2,507 to -0,1163	0,0314
Fruc 20+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	-1,062	-2,258 to 0,1333	0,0860
Fruc 40+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	0,2495	-1,060 to 1,559	0,9772

Abbreviations: GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; GPT: Gliadin digested with pepsin and trypsin

**Supplementary table 5:** Adjusted p-values of Tukey's post hoc test comparing difference in FITC permeation in Caco-2 cell monolayer after exposure to glucose and digested gliadin in different concentrations for one hour. Gliadin was pre-digested with pepsin trypsin and chymotrypsin (GPTC). For graphical representation see figure 2D.

<b>Tukey's multiple comparisons test</b>	<b>Mean Difference</b>	<b>95% confidence interval of difference</b>	<b>Adjusted P Value</b>
Medium vs. Glucose 20ug/mL	-0,4519	-0,8301 to -0,07370	0,0199
Medium vs. Glucose 40ug/mL	-1,594	-1,939 to -1,249	<0,0001
Medium vs. Gluc 20+ GPTC 0,5 mg/mL	-0,06888	-0,4471 to 0,3093	0,9812
Medium vs. Gluc 40+ GPTC 0,5 mg/mL	-1,088	-1,466 to -0,7101	<0,0001
Medium vs. GPTC 0,5 mg/mL	0,03308	-0,3122 to 0,3783	0,9990
Glucose 20ug/mL vs. Glucose 40ug/mL	-1,142	-1,487 to -0,7970	<0,0001
Glucose 20ug/mL vs. Gluc 20+ GPTC 0,5 mg/mL	0,3830	0,004821 to 0,7612	0,0470
Glucose 20ug/mL vs. Gluc 40+ GPTC 0,5 mg/mL	-0,6364	-1,015 to -0,2582	0,0025
Glucose 20ug/mL vs. GPTC 0,5 mg/mL	0,4850	0,1397 to 0,8302	0,0078
Glucose 40ug/mL vs. Gluc 20+ GPTC 0,5 mg/mL	1,525	1,180 to 1,870	<0,0001
Glucose 40ug/mL vs. Gluc 40+ GPTC 0,5 mg/mL	0,5058	0,1606 to 0,8510	0,0060
Glucose 40ug/mL vs. GPTC 0,5 mg/mL	1,627	1,318 to 1,936	<0,0001
Gluc 20+ GPTC 0,5 mg/mL vs. Gluc 40+ GPTC 0,5 mg/mL	-1,019	-1,398 to -0,6412	<0,0001
Gluc 20+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	0,1020	-0,2433 to 0,4472	0,8769
Gluc 40+ GPTC 0,5 mg/mL vs. GPTC 0,5 mg/mL	1,121	0,7761 to 1,467	<0,0001

Abbreviations: GPTC: Gliadin digested with pepsin, trypsin, and chymotrypsin; GPT: Gliadin digested with pepsin and trypsin





# NARRATIVE REVIEW: NUTRIENT DEFICIENCIES IN ADULTS AND CHILDREN WITH TREATED AND UNTREATED CELIAC DISEASE

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**Abstract:**

Nutrient deficiencies are well recognized as secondary consequences of celiac disease (CD) and closely related to the clinical presentation of affected patients. Despite their clinical significance, consensus is lacking on the pattern and frequency of nutrient deficiencies in CD, the usefulness of their assessment at the time of diagnosis and during follow-up. This review aims to provide an overview of nutrient deficiencies among pediatric and adult CD patients at diagnosis and on a gluten-free diet (GFD), and their potential causes in CD. Secondly, we review their impact on CD management strategies including the potential of nutrient supplementation. A search of Medline, Pubmed and Embase until January 2019 was performed. Despite a high variability between the reported deficiencies, we noted that nutrient deficiencies occur frequently in children and adults with CD at diagnosis and during treatment with a GFD. Both inadequate dietary intake and/or diminished uptake due to intestinal dysfunction contribute to nutrient deficiencies. Most deficiencies can be restored with (long-term) treatment with a GFD and/or supplementation. However, some of them persist while others may become even more prominent during GFD. Our results indicate a lack of comprehensive evidence on the clinical efficacy of nutrient supplementation in CD management highlighting the need for further studies.

**Keywords:** celiac disease; gluten free diet; nutrient deficiencies; nutritional status; supplementation

## 1. Introduction

Celiac disease (CD) is a common immune-enteropathy triggered by dietary gluten in genetically susceptible individuals [1]. In CD, the immunologic response to gluten peptides causes histological abnormalities in the small intestine. These histological aberrations such as villous atrophy reduce the functional capacity of the intestine [1]. A clinically relevant consequence is malabsorption resulting in an increased risk for nutritional deficiencies. These deficiencies can contribute to clinically important comorbidities such as anemia, osteoporosis and depression [2-4]. Nutritional deficiencies do not only play a role at the time of diagnosis, but also during treatment with a gluten-free diet (GFD).

The functional absorptive surface of the intestine in CD is expected to restore after treatment with a GFD, thereby reestablishing nutrient absorption. However, studies reveal that full histological recovery requires long-term treatment, especially in adult patients. This makes CD patients prone to nutrient deficiencies in the first period after initiation of a GFD, even when strictly adhering to the diet [5-8]. Another complicating factor that may trigger development of deficiencies while treating the disease with a GFD is the diet itself. Withdrawing gluten-containing foods from the diet and substituting them with cereals that are less rich in nutrients may lead to a nutritional imbalance contributing to an overall impaired nutrient status [9-11]. Currently, it is unclear which types of nutrient deficiencies are frequently present in CD patients at diagnosis and during treatment with a GFD. Moreover, there is a lack of knowledge on the clinical relevance of nutrient deficiencies and hence the potential impact of nutrient supplementation on the clinical outcome in CD. To answer the main research question that forms the basis of this narrative review, we reviewed the published scientific evidence for nutrient deficiencies in pediatric and adult CD patients at diagnosis and during treatment with a GFD. Moreover, the review provides an appraisal of underlying causes of deficiencies and the clinical relevance of nutrient supplementation. Currently, the clinical impact of nutrient deficiencies and the effectiveness of their treatment are currently poorly defined. Besides, the assessment of nutrient deficiencies can be executed by different approaches, including measuring the values of individual nutrients in blood or urine, or by indirect evaluation of putative clinical consequences of deficiencies. Both of the latter approaches are addressed in this review. Finally, the review aims to evaluate whether the currently available evidence is of sufficient quality to provide recommendations for clinical practice.

## **2. Materials and Methods**

For this narrative literature review, as defined by Grant et al., a search of Medline, Pubmed and Embase from 1960 until January 2019 was performed to identify potentially relevant publications [12]. The following search terms were used to search in titles and abstracts using “All fields”, as well as MeSH terms when available: ‘celiac disease’, ‘coeliac disease’, ‘nutrient status’, ‘dietary intake’, ‘vitamins’, ‘minerals’, ‘nutrients’, ‘gluten-free diet’, ‘histology’, and ‘histological recovery’. Additionally, a search with relevant nutrients was conducted including the search terms ‘iron’, ‘ferritin’, ‘vitamin D’, ‘vitamin B6’, ‘vitamin B12’, ‘calcium’, ‘folic acid’, ‘zinc’, ‘magnesium’. The search was limited to studies in the English language and full-text availability. This approach resulted in a total of 8478 articles for potential inclusion in this review. The search was not narrowed down further, to make sure as many eligible articles as possible were included in this overview. All potentially relevant original articles were screened for inclusion by two researchers in a step-wise approach; first, based on title, then on screening of the abstracts, and then by full-text screening of the remaining articles. Reference lists from the selected articles were also screened manually for relevant publications. The cited articles were selected based on the relevancy to the review objectives. Included were original studies on histological recovery in celiac patients on a GFD; nutrient intake of celiac patients; and nutrient deficiencies in untreated or treated celiac patients. As there is currently no clear consensus on the definition of nutrient deficiencies and how these should be evaluated, studies were included that measured serum nutrient values. Furthermore literature describing the link between nutrient levels and associated symptoms and comorbidities in CD were added to provide a general overview. Articles describing nutrient deficiencies were only included when they reported either the percentage or total number of patients with a nutrient deficiency. An appraisal of the quality of the studies was conducted to select articles of high quality which were summarized in the results of this review. The tables report the percentages of patients or of a reference population with nutrient levels below reference point and nutrient intake below recommendation (Table 1 and Table 2 respectively). For both the reference values for nutrient concentrations and reference values for recommended nutrient intake, the values chosen by the individual studies were used, meaning that the cut-off values vary between studies. Furthermore, this entails that no distinction was made in the severity of the nutritional deficit. This means, that the reported percentage of patients with a deficiency include those with severe deficiencies as well as sub-optimal values below the cut-off value. No selection was made based on the technique chosen to measure nutrient values and an overview of the selected methods was included when reported by the authors (see supplementary Table S1).

Only recent studies, not older than 15 years, were included in the tables. Furthermore, the study population had to be well defined with sound confirmation of CD diagnosis, as well as the moment of assessment, specifically differentiating between the moment of CD diagnosis and assessment of follow-up. Only those articles were selected that presented the results in a way that they could be extracted for this review. This included a clear differentiation between groups and definition of the moment of measurements. Furthermore, the results had to provide either percentages or numbers of subjects with nutrient deficiencies or insufficient intake of certain nutrients. Consequentially, articles only providing mean or median values of nutrient levels or nutrient intake within the groups were excluded. Single-case reports were not included. Level of evidence was assessed according to the 2009 Oxford Centre for Evidence-Based Medicine (OCEBM) [13,14]. The evidence level could be graded down based on the researchers' assessment of study quality and relevance and could be graded up in case of a large or very large effect size. An overview of all studies included in Tables 1 and 2 is provided in the supplementary material of this review. The overview includes subject characteristics and demographics of each study, as well as outcome parameters and details on the information provided by the authors concerning chosen diagnostic tests chosen and cut-off values for nutrient assessment. Lastly, the level of evidence was provided according to the 2009 OCEBM (see supplementary Table S1). Due to the small number of recent, high-quality publications on several nutrient values that met all the aforementioned criteria, other studies that did not meet these criteria were included in the review as well. These studies were included as the best available evidence and were highlighted within the text and tables as being of lower quality.

### **3. Nutritional Deficiencies in Celiac Disease at Diagnosis and on a Gluten-Free Diet**

An overview of the most important nutrient and mineral deficiencies reported in CD at diagnosis and on a GFD are summarized in Table 1. Percentages of nutrient deficiency in healthy reference populations are mentioned in Table 1 as well. The majority (69%) of the studies summarized in this review were conducted in Europe, 13% in North America, and 9% in India, the remaining studies being conducted in Israel, Australia and South America. Half of the studies were conducted prospectively, while the other half were mainly retrospective chart reviews or cross-sectional studies (see supplementary Table S1).

**Table 1:** Overview of nutrient deficiencies at diagnosis and during follow-up on a gluten-free diet.

Nutrient	Percentage of untreated CD patients with circulating levels below reference value (%)	Percentage of treated CD patients with circulating levels below reference value (%)	Percentage of treated CD patients with circulating levels below reference value (%)	Percentage of individuals in the general reference population with circulating levels below reference value (%)
				Short term follow-up, <2 years on a GFD (time on GFD)
<b>Adults</b>				
Iron (iron/ferritin)	6%-82% [15-23]	Serum iron: 44% Serum ferritin: 15% (1 year) [24]*	data not available	17% [23]
Vitamin D (25(OH)D)	5%-88% [18,20,25-28]	50% (1 year) [29]*	7.6% (5 years) 0% (mean 4 years for men, 9 years for women) [29,30]*	50% [25]
Calcium	0%-26% [17,19,20,28,31]	0% (1-2 years) [17]	data not available	data not available
Vitamin B12	5%-19% [16-18,20,22,32]	data not available	0% (8-12 years) [33]	7%-17% [18,23]
Vitamin B6 (vitamin B6/ Plasma pyridoxal 5 phosphatase)	15% [18]	data not available	37% (8-12 years) [33]	0% [18]
Folic acid (folic acid/folate)	11%-75% [15-20,22,23,31,32]	data not available	20% (8-40 years) [33]*	4%-14% [18,23,32]
Zinc	67% [18]	30%; (1 year) [24]*	20% (range 8 months-7 years) [34]*	data not available
Magnesium	13%-17% [35,36]*	data not available	data not available	data not available

Table 1: Continued

Nutrient	Percentage of untreated CD patients with circulating levels below reference value (%)	Percentage of treated CD patients with circulating levels below reference value (%) Short term follow-up, <2 years on a GFD (time on GFD)	Percentage of treated CD patients with circulating levels below reference value (%) Long term follow-up, >2 years on a GFD (time on GFD)	Percentage of individuals in the general reference population with circulating levels below reference value (%)
<b>Children</b>				
Iron (iron/ferritin)	12%-82% [22,23,37-39]	Serum iron: 5%-10% Serum ferritin: 21%-27% (6 months-2 years) [38,39]	Serum iron: 4%-8% (3-5.5 years) [38]	17% [23]
Vitamin D	0%-70% [25,38-42]	0%-57% (6 months-2 years) [38-41]	12%-25% (2-5.5 years) [38]	4%-30% [25,40,42]
Calcium	0%-41% [37,38,40,43-46]	0% (6 months-2 years) [38,40]	0% (3 years-5.5 years) [38]	0% [40]
Vitamin B12	1%-14% [22,23,37,38]	0%-1% (6 months-2 years) [38,39]	0% (3-5.5 years) [38]	7% [23]
Vitamin B6	data not available	data not available	data not available	data not available
Folic acid	14%- 31% [22,23,37,38]	0%-3% (1-2 years) [38]	0% (3-5.5 years) [38]	14% [23]
Zinc	19%-72% [37,39,47]	16%-18% (6-18 months) [39]	data not available	data not available
Magnesium	7%-11% [40,48]	data not available	4% (11 years; range 3-17) [48]	0% [40]

Overview of reported percentages of adult and pediatric CD patients with a nutrient deficiency at the moment of diagnosis and during follow-up with a GFD. All reported values are summarized in ranges, with the corresponding studies referenced in square brackets. The duration of GFD is mentioned in brackets in columns three and four. \* Studies that did not meet quality criteria were only included if no other eligible article existed for that nutrient level and are marked by an asterisk.

Abbreviations: celiac disease (CD); gluten-free diet (GFD).

### *3.1. Nutritional Deficiencies at Moment of Diagnosis in Untreated Celiac Disease*

Twenty-nine studies that evaluated nutritional deficiencies in CD at diagnosis were identified, only 15 of which describe nutritional status in children. The most frequently described deficiencies in CD patients at diagnosis are of iron, vitamin D, calcium, vitamin B12, folic acid and zinc. Presence of iron deficiency was described in 6%–82% of adult patients and in 12%–82% of pediatric patients newly diagnosed with CD [15–23,37–39]. Iron status has been well researched in regard to CD and the results presented here were obtained from twelve different study populations.

Vitamin B12 (cobalamin) deficiency has been reported in 5%–19% of untreated CD patients [16–18,20,22,23,32,37]. Folic acid deficiency (vitamin B9) has been described in 11% up to, as many as 75% of adults with untreated CD and 14%–31% of children [15–20,22,23,31,32,37,38].

The prevalence of deficiencies in several micronutrients in CD is unclear. This is due to lack of scientific evidence or high variety between studies. This is the case in vitamin B6 deficiency, for instance, which has recently only been studied in two adult but no pediatric CD cohorts. A recent study from Wierdsma et al. showed that vitamin B6 deficiency was present in 15% of untreated adult CD patients but not in controls [18]. In contrast, other studies reported similar vitamin B6 levels in adult CD patients compared to healthy controls [49].

Vitamin D levels were demonstrated to be low in 5%–88% of untreated adult patients, and in 0%–70% of untreated pediatric patients [18,25–27,40–42,45]. Circulating levels of calcium were described to be low in 0%–26% of untreated adults and 0%–41% of pediatric patients [17,19,37,40,45,46]. The only prospective case-control study of good quality investigating hypocalcemia was conducted by Zanchi et al. which found a high prevalence of 40.7% of hypocalcemia at diagnosis in pediatric CD patients, compared to 0% in controls [40]. Similarly, the evidence of higher quality available for vitamin D levels also indicates a high prevalence of vitamin D deficiency in pediatric CD patients. Tokgöz et al. found a prevalence of vitamin D deficiency in 92.4% of CD patients compared to 18% in controls. This difference becomes even more profound when separately investigating vitamin D insufficiency (levels below 30 ng/mL) which was present in 61.5% of CD patients and merely 4% of healthy controls [42].

Next to calcium, deficiencies in other minerals and elements are associated with CD, although literature reporting on these is particularly scarce. Magnesium deficiency was reported in 7%–11% of untreated pediatric CD patients [40,48]. Magnesium deficiency in untreated adult CD patients has only been reported in two studies that did not meet the criteria for the quality assessment, being published longer than 20 years ago, indicating a prevalence of magnesium deficiency in 13%–17%

[35,36]. Zinc deficiency has been found in 67% of untreated adult patients. Notably, all three studies reporting zinc levels in pediatric patients with active disease have found levels below the reference value in more than half of the patients, and a prospective randomized controlled trial conducted by Rawal et al. even found zinc deficiency to be present in more than 70% of patients [37,47,50].

Taken together, nutrient deficiencies are highly prevalent at time of CD diagnosis in the pediatric and adult population, although there is a variance in reported fractions of nutrient deficiencies. Importantly, not only routinely measured nutrients such as iron and vitamin B12 are deficient, but also less recognized and studied nutrients such as zinc appear to be deficient frequently in patients with active CD.

### *3.2. Nutritional Deficiencies while on a Gluten-Free Diet in Treated Celiac Disease*

After diagnosis and institution of a GFD, restoring the nutrient status and maintaining adequate nutrient intake in CD patients remains a challenge. Studies reported that mean micronutrient levels increase in adult patients within one year of gluten elimination [17,23,32]. However, the velocity of this increase can vary, and an increase in nutrient concentrations does not always lead to full normalization. The rate of normalization as well as the time period necessary for nutrient levels to restore is of clinical importance, as it can impact health and disease outcomes in the short and long term. The selected studies mainly chose a time period of 6 to 12 months after institution of the GFD as indicators for short-term follow-up. Based on these results, few conclusions can be drawn on the time period necessary for recovery of nutrient deficiencies after diagnosis (see Table 1). When studying the prevalence of nutritional deficiencies in CD after diagnosis, these can be grouped into three categories: nutrient deficiencies that generally fully normalize during treatment; deficiencies that are more prevalent during follow-up than at the moment of diagnosis, and lastly, nutrients of which levels generally improve, yet not to the extent seen in a healthy reference population.

Nutrients that appear to be generally corrected in CD patients on a GFD are vitamin B12, folic acid, calcium and magnesium (see Table 1). Vitamin B12 deficiency has been described in 0% of treated adult CD patients by Vilppula et al. [17].

A GFD seems to also improve or even normalize folic acid levels in CD patients [17,23,24,30,32,51,52]. Folic acid deficiency in adults with CD on a GFD has not been reported in a study that met the quality assessment of this review, but it has been described in studies with a moderate quality. Hallert et al. reported low serum folate levels in 20% of adult CD patients on a 10-year GFD with a normalized intestinal mucosa in a study that met all criteria of our quality assessment except for the year of publication which was more than 15 years ago [33]. The prevalence of folic acid deficiency in treated children with CD has been studied in one

recent publication that met our quality assessment. Wessels et al. describe a low prevalence of 0%–3% in their retrospective pediatric cohort on a GFD [38].

Likewise, resolution of calcium deficiency and to a lesser degree vitamin D levels was observed after institution of a GFD in most cases, which was confirmed in the study by Zanchi et al. with a high level of evidence [17,29,30,38–41,53]. Calcium levels have been described to be normal in all treated children with CD, while vitamin D was low in 0%–57% of children and in 0%–50% of adult patients on a GFD [29,30,38–41,53]. Next to the quality of the studies, it is important to consider that vitamin D levels especially are also low in the general population, when interpreting these results [25,40]. Additionally, the studies report nutrient deficiencies in the serum and do not take distribution and storage in the body into account. In the case of calcium and vitamin D for example, bone mineral density (BMD) and dental health are also a measure for total calcium in the body. Comorbidities associated with CD-related nutrient deficiencies are reviewed in the next section of this review.

The effect of a GFD on magnesium levels is scarcely studied, yet appears to result in normalization of magnesium levels. Rujner et al. showed that the frequency of magnesium deficiency in treated adult CD patients (median 11 years on a GFD) was similar to that in untreated patients and controls [48]. In children, magnesium deficiency has been described in 4% of celiac patients on a GFD for 11 years [48].

In contrast to the aforementioned nutrient deficiencies that generally restore, other deficiencies might be even more prevalent on a GFD compared to the moment of diagnosis. Vitamin B6 deficiency might be an example of this. Studies reporting vitamin B6 levels in CD are, however, scarce and no study was available that met the inclusion criterion of this review which only included studies not older than 15 years. In a Swedish patient cohort of moderate quality published in 2002, low vitamin B6 plasma levels were found in 37% of adult CD patients on a long-term GFD, despite a recovered intestinal mucosa and dietary intake meeting recommendations [33].

Lastly, a considerable group of micronutrient imbalances improve, yet remain a prevalent problem on a GFD, iron deficiency being a chief example. Iron deficiency was described in 14%–41% of adult celiac patients on a GFD [54]. Strikingly, little evidence is available on the prevalence of iron deficiency in pediatric patients on a GFD and even less on the potential clinical implications in this regard. This should be given more attention, considering that iron deficiency anemia (IDA) is one of the most prevalent extra-intestinal manifestations of CD at diagnosis [21,38,55]. Adherence to a GFD does appear to reduce the prevalence and severity of IDA and increases serum iron and ferritin, although repletion of iron stores may take a

prolonged time after healing of the intestinal mucosa [17,24,43,51,56,57]. In adult CD patients, the degree of histological recovery was associated with an increase in serum hemoglobin concentrations and impaired hemoglobin levels were evident in patients with incomplete mucosal recovery [57]. In pediatric CD patients on a GFD, the severity of villous atrophy correlated inversely with serum ferritin level, although no correlation with other hematological parameters was found [58]. Popov et al. showed a significant increase in mean serum ferritin concentration in pediatric CD patients after GFD in a retrospective chart review [59]. These findings were independent of the use of iron supplementation. Likewise, Wessels et al. showed a decrease in the proportion of pediatric CD patients with an iron deficiency after institution of a GFD [38,59]. In summary, iron deficiency appears to restore in a substantial proportion of CD patients after institution of the GFD. However, iron deficiency is still present in a considerable fraction of CD patients on a GFD.

In a similar way to iron deficiency, zinc deficiency appears to not improve sufficiently during CD treatment. A GFD has been shown to reduce zinc deficiency from a proportion of up to 72% of untreated CD patients to 16%–30% of patients on a GFD presenting with this deficiency [18,24,34,37,39,47,60]. Although zinc appears to restore in most patients, zinc deficiency was still found in 20%–30% of adult CD patients on a GFD [24,34].

When looking at hyperhomocysteinemia, controversial results have been found, and it is unclear whether this is generally corrected or remains a relevant consequence of CD even on a GFD. Studies on the occurrence of hyperhomocysteinemia showed a decrease of homocysteine levels after starting a GFD [32,49]. Dickey et al. reported normal levels in CD patients with recovered villous atrophy, while patients with persistent villous atrophy tended to have mildly elevated homocysteine levels [49]. However, Hallert et al. showed raised homocysteine levels in 17% of adult CD patients on a strict GFD for several years [33,61]. Also, levels of serum folate, vitamin B12 and vitamin B6 correlated inversely with homocysteine levels in CD patients [32,33,49,61]. Yet, Hadithi et al. reported that the presence and severity of CD were determinants of homocysteine levels, independent of measured B vitamin status [62].

### *3.3. Comorbidities Potentially Related to Nutrient Deficiencies in Celiac Disease*

Nutrient deficiencies in CD can be the cause or a contributing factor to several symptoms and comorbidities associated with CD (see Figure 1). These, symptoms and comorbidities can serve as indicators for nutrient deficiencies in the long and short term.

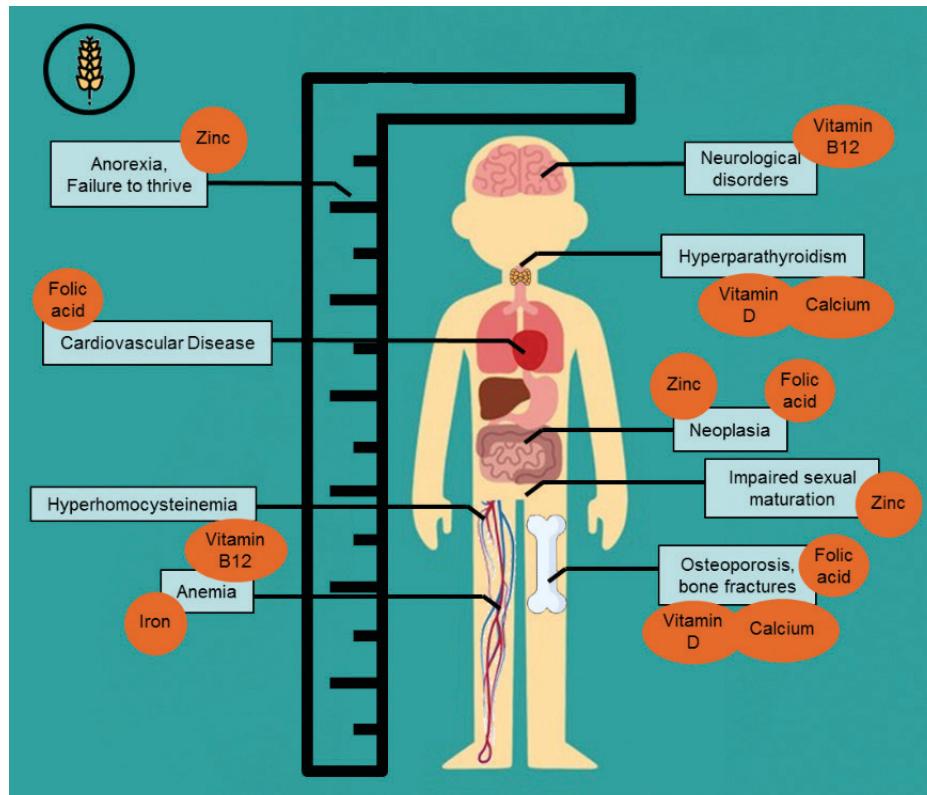


Figure 1. Comorbidities associated with nutrient deficiencies often found in celiac disease.

IDA is one of the most prevalent extra-intestinal manifestations of CD at diagnosis [21,55]. IDA can cause symptoms associated with CD such as fatigue, headaches and decreased exercise tolerance. Anemia in CD has further shown to be associated with greater disease severity and slower histological recovery in response to the GFD [63–65].

Other plausible causes of anemia in CD include vitamin B12 and/or folate deficiency, two nutrients essential in DNA synthesis. Deficiencies in these nutrients can limit DNA synthesis and result in megaloblastic anemia [66,67]. Vitamin B6 is important in hemoglobin formation, hence low levels of vitamin B6 can also contribute to the development of anemia in CD [68]. Therefore, anemia and its resulting symptoms can be a useful indicator for the importance of nutrient deficiencies in CD.

Both vitamin B12 and folate, as well as vitamin B6 are also essential in the conversion of the harmful homocysteine. B vitamin deficiencies can therefore cause hyperhomocysteinemia in CD [66,67,69–71]. Consequentially, increased severity of celiac lesions is associated with higher homocysteine levels [62]. Aberrant

homocysteine levels are related to an increased risk for venous thromboembolism, vascular disease, osteoporosis and adverse pregnancy outcomes [72–74].

Vitamin B12 deficiency has further been held responsible for several neurological symptoms which have been described in CD [66]. Folic acid deficiency has also been linked to a wide range of neurological disorders and conditions as diverse as neoplasia, cardiovascular disease and osteoporosis [67].

Other complications related to CD involve bone health, which is closely related to the nutritional status especially of calcium and vitamin D [75]. Impaired bone health is highly prevalent in children and adults at diagnosis of CD, even in individuals with mild enteropathy (without villous atrophy) [43,44,76–85]. In adult onset CD, a GFD generally improves but rarely normalizes BMD and the serious impact of this is shown in studies examining fracture risk [51,86]. Jafri et al. showed that fracture risk is increased in CD at diagnosis and during long term follow-up and a meta-analysis by Heikkilä et al. confirmed this increased risk for fractures in patients with CD [87,88].

In pediatric CD, the evidence on recovery of bone health and its impact on growth and long-term complications is more divergent. A strict GFD promotes an increase in BMD that may lead to complete recovery of bone mineralization within 1–2 years [40,43–45,77–82]. For instance, Mora et al. found that BMD and bone area normalized within a year of treatment in pediatric CD patients [78,79]. However, Kalayci et al. found that one year of strict GFD was insufficient for osteopenia to resolve in a substantial number of pediatric patients and suggests that BMD should be monitored in these children [78]. The influence that this could have on bone health in later life is uncertain. Moreover, the long term consequences of an initial impaired bone health and low calcium and vitamin D levels during childhood, regardless of its restoration, are not clear.

Two other important minerals that can cause complications in CD patients when deficient, are zinc and magnesium. As summarized earlier, zinc deficiency is highly prevalent at diagnosis in pediatric and adult CD patient and seems to restore insufficiently when on a GFD [18,24,34–37,43,47,50,89].

Zinc is an essential trace element involved in numerous enzymatic reactions, biochemical functions and immune responses, and is required for growth and cellular function [90–92]. Zinc deficiency can alter protein synthesis, and in the absence of zinc growth retardation and impairment of sexual maturation occurs, which makes it particularly important for the pediatric CD population [92,93]. Classical presenting symptoms of CD such as anorexia and failure to thrive have therefore been linked to zinc deficiency [94]. Altuntas et al., found that zinc deficiency was present in more than half of the patients presenting with short stature that were diagnosed with

CD in a pediatric population [50]. And although zinc deficiency appears to remain a relevant problem after institution of a GFD, little is known about the consequences of transient or persistent zinc deficiency in CD [47,89,94].

### *3.4. Role of Nutrient Supplementation in Celiac Disease Management*

Overall, only few studies have systematically assessed the impact of nutrient supplementation as an additional therapy next to a GFD in CD. Some studies have suggested that nutrient supplementation may positively influence recovery, whereas others describe no difference between patients receiving supplementation and patients who do not.

Foods rich in iron can be recommended in patients on a GFD, and supplements may be prescribed in clinical practice [9–11]. However, two prospective studies published in 1996 and 2003 found that even after iron supplementation for up to one year in pediatric CD patients, a significant number continued to have iron deficiency [43,58]. Additionally, a retrospective chart review showed that improvement of iron status in pediatric CD patients on a GFD was unrelated to the intake of supplementation [59]. It must, however, be noted that the use of supplementation was based on parent-reported medication history.

In a study of vitamin status in adult CD patients after long-term treatment, circulating vitamin B12 as well as B6 levels were poorly correlated with dietary intake [33]. By contrast, vitamin B supplementation (folic acid, cyanocobalamin and pyridoxine) in CD patients on a long-term GFD has been shown to be effective in increasing serum vitamin B12 and B6 levels as well as serum folic acid levels [61,62]. Median serum levels were significantly higher in CD patients on B vitamin supplementation compared to both CD patients not using supplementation and healthy controls [62]. However, the role of each individual vitamin has not been established.

Supplementation of vitamin B12 in patients on a GFD may be effective to prevent neurological complications associated with CD [95]. An increase in serum folic acid due to supplementation has been shown to be clinically relevant, since 6 months of supplementation improved anxiety and depressed mood in patients with longstanding treated CD [61].

Complications of poor bone health have far-reaching effects on a lifelong scale. Therefore additional measures improving bone health or at least preventing further bone loss in CD patients on a GFD might be beneficial. Calcium and vitamin D supplementation is considered a possible treatment option. However, neither vitamin D supplementation alone nor combined calcium and vitamin D supplementation for one year in adult CD patients provided additional benefit to the GFD with respect to

BMD [96,97]. In children and adolescents with CD on a GFD, two years of combined calcium and vitamin D supplementation was reported to increase BMD, but levels did not normalize to those measured in healthy controls [98].

Supplementation in a methodologically sound randomized controlled trial of zinc for four weeks in newly diagnosed pediatric CD patients was not effective in increasing zinc levels compared to children who started a GFD without supplementation [47]. To our knowledge, the effect of supplementation of magnesium in CD patients has not been studied.

#### **4. Causes of Nutritional Deficiencies in Celiac Disease**

As shown above, nutritional imbalances are present in a substantial number of patients diagnosed with CD [99–101]. Nutrient deficiencies result from an imbalance between nutrient supply and biological need. In CD, nutrient supply can be insufficient due to impaired uptake on the one hand, and as a consequence of inadequate nutrient intake when on a GFD on the other hand. Impaired uptake is the main factor of nutrient deficiencies at diagnosis and becomes less important after histological recovery. After institution of a GFD, recovery of the small intestine occurs in most CD patients, making insufficient nutrient intake the factor most likely to contributing to nutrient imbalances, due to possible nutritional inadequacy of the GFD itself (see Table 2) [102–106].

**Table 2:** Overview of dietary intake of nutrients on a gluten-free diet in celiac disease patients and reference populations.

Nutrient	Percentage of CD patients with nutrient intake below recommendations (%)	Percentage of individuals in the general reference population with nutrient intake below recommendations (%)
<b>Adults</b>		
Iron	46%–54% [52,99,101,104,107]	14% [52,99]
Vitamin D	53%–100% [99,107,108]	data not available
Calcium	12%–78% [99,101,104,107–110]	6%–29% [52,99]
Vitamin B12	10%–61% [99,107,111]	1%–65% [99,111]
Vitamin B6	33% [111]	17% [111]
Folic acid	35%–100% [54,101,107,110–112]	3%–100% [52,99,111]
Zinc	11%–58% [52,101,107,108,110]	30% [52]
Magnesium	28%–50% [52,101]	29% [52]

**Table 2: Continued**

Nutrient	Percentage of CD patients with nutrient intake below recommendations (%)	Percentage of individuals in the general reference population with nutrient intake below recommendations (%)
<b>Children</b>		
Iron	8% [113]	43%–79% [100,110]*
Vitamin D	68% [113]	data not available
Calcium	8%–54% [48,113]	86% [110]*
Vitamin B12	0% [113]	data not available
Vitamin B6	8% [113]	data not available
Folic acid	80% [110]	57% [110]*
Zinc	40% [110]	43% [110]*
Magnesium	29%–76% [48,113]	data not available

Overview of reported percentages of adults and children with CD and of general reference populations with nutrient intake below the recommended levels. All reported values are summarized in ranges, with the corresponding studies referenced in square brackets. \* Studies that did not meet the quality criteria were only included if no other eligible article existed for the intake of that nutrient and are marked by an asterisk. Abbreviations: celiac disease (CD).

#### 4.1. Impaired Absorption due to Compromised Intestinal Epithelial Function

Absorption of macro- and micronutrients occurs in the small intestine, the site specifically affected in CD. Therefore, nutritional deficiencies due to diminished absorptive capacity are expected in active CD. Villous architecture and absorptive cells (enterocytes) are severely damaged and reduced in number in CD patients. Additionally, many enzymes necessary for digestion and absorption of nutrients are depleted or dysfunctioning, particularly brush border enzymes like the disaccharidases maltase, isomaltase, and lactase [114]. The disease affects the proximal small intestine and extends distally for a variable length in a more or less continuous fashion, with more severe damage in the proximal than in the distal part [54]. Consequently, mucosal impairment in CD at diagnosis may result in malabsorption of macro- and micronutrients, and studies show that more pronounced mucosal damage leads to increasing nutritional deficiencies [19,115,116].

The proximal small intestine is the site of absorption for iron, folate, and calcium [117]. Minerals and elements including zinc and magnesium are also mainly absorbed in the proximal small intestine, explaining why their levels may be low in CD patients [34]. Deficiencies in these nutrients can be explained by reduced

absorptive capacity as well as reduced enzyme function in the affected proximal small intestine.

Iron deficiency in untreated celiac patients is primarily caused by malabsorption due to loss of duodenal enterocytes. This results in a reduction of absorptive surface, as well as decreased amounts of the brush border enzyme ferrireductase, necessary for iron transport across the cellular membrane [117,118]. Moreover, enterocytes have an iron-storage capacity themselves which is directly affected by villous atrophy [117,118]. Although it has been previously suggested that gastrointestinal blood loss might also contribute to iron deficiency, recent studies suggest gastrointestinal bleeding to be uncommon in CD [119,120].

Diminished enzyme count and function also play a role in folic acid deficiency in untreated CD, since dietary folate, in the form of polyglutamates, is absorbed and cleaved to the monoglutamate form in the duodenum and jejunum, which are affected in CD [121].

Another example highlighting the complex causal cascade involved in development of nutrient deficiencies in CD is calcium. Potential factors involved are: loss of villous surface area, binding of calcium to unabsorbed fatty acids in the intestinal lumen, impairment of the active intestinal calcium transport mechanism due to depletion of vitamin D in enterocytes, and decreased dietary calcium and vitamin D intake secondary to concomitant lactase deficiency [122–124]. This shows that, for example, vitamin D deficiency can in turn enhance or cause other deficiencies, for example of calcium and magnesium. The small intestine normally absorbs up to 50% of magnesium intake, in part under the influence of active vitamin D [125,126].

More distal parts of the small intestine, jejunum and proximal ileum are responsible for absorption of carbohydrates, fat, vitamin B6 and fat-soluble vitamins (A, D, E, and K), while vitamins B12 absorption occurs in the terminal ileum [127]. Given the relative absence of villous atrophy in the (terminal) ileum, the mechanisms responsible for vitamin B12 deficiency in CD are unclear. In the ileum, vitamin B12 is absorbed bound to intrinsic factor, while a small portion is absorbed via passive diffusion along the entire small intestine. Hypothesized causes for vitamin B12 deficiency in CD include decreased secretion of gastric acid, dysfunctioning intrinsic factor, autoimmune gastritis, bacterial overgrowth or decreased efficiency of mixing with transfer factors [66,95,128].

#### *4.2. Histological Recovery on a Gluten-Free Diet*

Strict adherence to a GFD has classically been assumed to result in complete recovery of the intestinal mucosa and its absorptive function [129]. Indeed, elimination of gluten from the diet improves villous architecture and reduces the

number of intraepithelial lymphocytes (IEL) [129]. However, despite its simple appearance, it is difficult to adhere to a strict GFD in the Western world where wheat is widely present in food (see 4.3). Inadvertent gluten intake may contribute to delayed mucosal recovery, as well as contamination of gluten-free products. Nevertheless, studies have shown that complete histological normalization is not always achieved in adult patients even when they maintain a strict GFD [5,7,129–132]. Currently, there is no full agreement on this topic. Therefore, insufficient histological recovery in CD is a potential reason for inadequate restoration or development of nutrient deficiencies even on a GFD and the mechanisms described above remain relevant in explaining causes of deficiencies in treated CD (see 4.1). In contrast, full histological recovery is almost always found in pediatric CD patients with good compliance to the GFD [8,133]. Moreover, the progress of histological recovery seems faster in children than adult CD patients [8,133].

Therefore, reduced absorptive capacity appears to be a less important cause of nutrient deficiencies in pediatric CD patients on a GFD [134]. Several studies performed to evaluate the histological recovery in adult CD patients after initiation of a GFD report a minority of CD patients achieving complete recovery of the intestinal mucosa within the first years of treatment. Long-term studies suggest that villous atrophy persists in 4%–79% of adult CD patients despite gluten elimination for an average of 8 years [5,7,129–131].

The wide range in reported histological recovery rates may be caused by the lack of prospective studies (selection bias), mixed adult and pediatric populations, various degrees of dietary strictness, a variable duration of gluten elimination prior to follow-up biopsies, and different definitions of mucosal and villous recovery. Nevertheless, authors agree that recovery of the celiac mucosa requires long-term treatment, especially in adult patients [6,7,129,130].

The slow and incomplete intestinal recovery in CD patients on a GFD might contribute to nutrient deficiencies. For instance, the degree of histological damage in CD patients has been shown to correlate with the severity of iron deficiency at time of diagnosis [18,19,24,27]. In addition, folic acid levels are generally depressed in patients with severe villous atrophy compared to patients with milder lesions. Although most studies reported no significant differences in vitamin B12 concentrations between patients with partial or (sub)total villous atrophy, others showed that vitamin B12 levels tended to correlate with the severity of the intestinal lesions [23,24,27,95,116,128]. However, no correlation has been found to date between the severity of the small intestinal lesion and vitamin D, B6, calcium, magnesium and zinc levels in celiac patients.

#### 4.3. Nutrient Imbalance associated with a Gluten-Free Diet

Historically, the treatment of CD has focused on the avoidance of gluten-containing food and less importance has been devoted to the nutritional quality of the GFD [99]. However, maintaining a nutritionally adequate diet on a GFD requires effort and attention and insufficient dietary intake of nutrients is an important contributing factor to nutritional deficiencies in CD. The grains and gluten-containing products excluded in a GFD, are a major source of iron, dietary fiber, B vitamins and iodine, and contribute substantially to the energy and protein content of a normal diet. Their elimination from the diet inevitably alter the macro- and micronutrient composition of a GFD [33,52,56,99,106,135].

Rice, corn and potatoes are widely used as natural substitutes of gluten-containing grains but are generally less nutrient dense. Moreover, processed gluten-free products are often of lower quality and poorer nutritional value compared with their gluten-containing equivalents [9–11,106]. A number of nutrient-dense grains, including the pseudo-cereals buckwheat, quinoa and amaranth, represent a safe alternative concerning gluten absence while improving the variety and palatability of the GFD, and are a good source of carbohydrates, protein, dietary fiber, vitamins, minerals, and polyunsaturated fatty acids [136,137].

On a macronutrient level, several studies suggest that a GFD is characterized by lower intake of (complex) carbohydrates and fibers, with subsequent higher protein and fat consumption [30,33,52,56,99,108,113,135,138]. The altered macronutrient dietary patterns can have negative consequences. For example, poor dietary fiber intake can increase the risk of other chronic diseases [99,101,113,135,139]. Additionally, a rise in body mass index (BMI) and increased prevalence of metabolic syndrome has been observed in CD patients after initiation of a GFD [140,141].

#### *Intake of Micronutrients*

Gluten-free cereal products generally contain inferior amounts of iron, folate and B vitamins compared to the products they are intended to replace, contributing to a lower intake of these nutrients by CD patients on a GFD [9–11]. Adult CD patients on a GFD failed to achieve the recommended daily amount of iron intake in 46%–54% of cases (see Table 2) [52,99,101,104,113,135,138]. In contrast, other studies suggested that iron intake is similar or even higher compared to healthy controls and Ohlund et al. found that merely 8% of pediatric CD patients failed to meet the recommended intake for iron [52,99,101,135,138]. Likewise, the intake of folic acid in adult CD patients is generally below recommendations (in 35%–98% of patients), but was shown to be similar or higher compared to the reference population in most studies [33,49,52,99,101,113,138]. In children with CD, folic acid intake has been shown to meet recommendations, similar to controls [138]. Additionally, the correlation between dietary intake and plasma levels of folate was found to be

poor [33]. Vitamin B6 intake has been reported to be below recommendations in 33% of adults, which was significantly higher compared to 17% in controls in a cross sectional case-control study by Valente et al., and in 8% of pediatric patients adhering to a GFD [111,113]. A low nutrient density of the other B vitamins thiamine (B1), riboflavin (B2) and niacin (B3) has been found in the diet of treated pediatric CD patients compared to healthy controls [113].

Surprisingly, the intake of nutrients not present in cereal products appears to be low as well in patients on a GFD. Animal products (meat and dairy) represent the only dietary source of vitamin B12 and are not restricted in a GFD. Therefore, the intake of vitamin B12 in CD patients on a GFD appears to meet recommendations, both in adults and children [33,113,138]. However, Kinsey et al. showed a low mean daily vitamin B12 intake in 10% of patients aged above 65 [99]. The intake of vitamin D has been suggested to be below recommendations in both pediatric patients (68% with a poor intake) and in adult patients (53%–100%) on a GFD, even below the generally low intake in healthy subjects [99,113,138].

Although minerals are located in the germ of grains and are not expected to be completely lost during the refining process, there is evidence that suggests low intake of minerals in CD patients on gluten elimination. Due to secondary lactose intolerance, calcium consumption might be low in CD patients. The intake of calcium in CD patients adhering to a GFD failed to achieve recommendations in 12%–78% of adults and in 8%–54% of children, although it has been shown to be similar or above intake-levels in reported reference populations [48,52,99,101,104,107–110,113]. Low calcium intake has a negative effect on BMD, which can already be impaired at time of CD diagnosis. Patients on a GFD also failed to achieve the recommended daily amount of magnesium intake in 29%–76% of children and in 28%–50% of adults, in line with the general population [48,52,101,113,138]. Furthermore, zinc intake was reported to be below recommendations in 11%–58% of adult CD patients adhering to a GFD, in line with intake in healthy adults in the reference population [52,101,108]. Pseudo-cereals contain higher amounts of minerals compared to other gluten-free cereals such as rice and maize and, therefore, provide a good alternative for CD patients [142].

Nutrient inadequacies in processed gluten-free products and incorrect dietary choices may contribute to inadequate nutrient intake identified in CD patients adhering to a GFD. In line with this, nutrient imbalances were more evident in children with strict compliance to a GFD than non-compliant patients [100]. A different choice of grains has the potential to improve the nutrient profile of the diet for individuals with CD, but attention must be paid to the complete dietary pattern [137]. Taken together, attention is required to the dietary shortcomings of micronutrients in CD patients adhering to a GFD.

## 5. Discussion

The current review of the available literature indicates that both newly diagnosed and CD patients following a GFD frequently exhibit nutrient deficiencies. Second, nutrient deficiencies are associated with short- and long-term manifestations and complications of CD [2–4,49]. However, the clinical impact of nutrient deficiencies and their modulation by treatment in CD patients are still unclear based on the available literature. Our current review identifies a high degree of variation between reports and a deficit in scientific evidence supporting a consistent role of nutrient deficiencies in CD on several levels. For example, no high-quality studies were identified that assessed deficiencies of iron, vitamin D, vitamin B12, folic acid, zinc or magnesium status in adult CD patients on a GFD. In general, after the analysis of the level of evidence of the reports reviewed in this work, only low- to moderate-level studies and practically no high-quality, high-level evidence studies were available. As a result, the evidence base is not strong enough to pronounce sound recommendations on CD management concerning nutrient status. Therefore, we suggest evaluating nutrient levels at the time of diagnosis as well as regularly during follow-up, until further research provides the evidence base necessary to generate more detailed recommendations. The results of this review show the importance of a nutritionally balanced dietary pattern as a part of CD management that can still lead to clinical improvement in most patients. Health providers should focus not only on gluten avoidance, but also on a balanced diet with respect to macro- and micronutrients in all CD patients. Early education of patients by a skilled dietitian with expert knowledge in CD is needed to address the achievement of adequate (micro) nutrient intake in patients on a GFD. Naturally gluten-free foods should be recommended as these have a higher nutritional value in terms of protein and fiber provision and vitamin content as opposed to the commercially purified gluten-free products [9–11]. Fortification of gluten-free foods needs to be considered and should at least match the micronutrient content of the foods they intend to replace. Targeted supplementation based on laboratory findings or a multivitamin and mineral supplement could be beneficial to the health status and recovery of individual CD patients, although care should be taken to avoid hypervitaminosis. Thus, vitamin B12 as well as folic acid supplementation have been shown to be clinically relevant in preventing or reducing neurological and psychological symptoms in CD patients. In children and adolescents, vitamin D and calcium supplementation are of particular importance to improve BMD. Interestingly, most studies evaluating the role of dietary supplements in CD management mainly focus on the restoration of blood levels as opposed to the effects on clinical consequences of nutrient deficiencies. However, if clinically relevant outcomes are investigated, the focus lies largely on long-term effects of nutrient supplementation on parameters such as BMD. Thus, short-term adverse effects of nutrient deficiencies are not taken into account. IDA and iron deficiency

for example, may improve on a GFD regardless of the use of iron supplements, but may improve faster in patients using supplementation. A prolonged duration of recovery could have potentially harmful effects on the patients' health and/or psychosocial well-being. A prolonged period of IDA in pediatric CD patients could, for instance, result in a longer period of fatigue and weakness, impairing school performance and social life. In adult CD patients, this burden could also have an additional societal impact when resulting symptoms lead to reduced work performance or prolonged sick leave [143,144]. Taking this into account, the time it takes for nutrient deficiencies to restore after initiation of GFD treatment should be of interest in future studies. Moreover, efforts should be made to generate more evidence supporting sound recommendations regarding the effects of treatment and monitoring of CD patients in which a nutrient deficiency or a clinical consequence of deficiency has been detected.

As mentioned above, certain limitations of this review's methodology should be taken into account. These limitations, combined with several weaknesses in the current literature, especially concerning the assessment of nutrient deficiencies, should be addressed and summarized. This is not only of great importance for interpretation of the presented overview, but also to provide recommendations concerning future research efforts. Although this narrative review has not been constructed as a systematic review, an extensive literature search of three databases has been conducted aiming to give as comprehensive an overview as possible. Nevertheless, this review is not a complete representation of all studies, but rather provides an overview of the current state of knowledge. High-quality studies were also included in this review, however, due to their scarcity some studies of lesser quality were considered in order to provide a general overview (see supplementary Table S1). Through conducting the literature search and selection by two investigators and broad screening for eligible articles, we aimed to minimize the risk of selection bias, but nonetheless we still present a narrative review.

A lack of consensus could be identified concerning several methodological aspects. This results in high variability in conducted studies concerning patient selection, chosen cut-off points for nutrient values and varying quality of assays measuring nutrient levels. Therefore, we present the reported percentages of patients with micronutrient values below the reference points, as chosen by the researchers of the respective studies. A uniform, clinically relevant cut-off value was thus not selected, as this can be controversial regarding several nutrients, for instance concerning vitamin D concentrations [145–147]. Another relevant point of concern is whether micronutrient levels measured in the blood (serum or plasma) are an accurate indicator of the overall status of a certain micronutrient in the entire body. This relates, for example, to zinc and magnesium measurements. It was suggested that other biomarkers in the blood could be a more accurate indicator of zinc status

in the body [148]. Similarly, there are conflicting views on the use of magnesium levels in blood or urine as an assessment for magnesium status [149]. Notably, few studies make a distinction in the severity of nutrient deficiencies, but rather only report on the number of patients not meeting the lower cut-off value (see supplementary Table 1) [40–42]. In order to truly evaluate the scope of the problem of nutrient deficiencies in CD, future studies might separately report on patients with sub-optimal nutrient values and patients with severe deficiencies. Overall, these shortcomings reflect general limitations of the current literature on this topic and could be viewed as an important outcome of this review. A consensus on these methodological issues should thus be achieved before studies assessing the subject matter elaborated upon in this review are executed.

## **6. Summary and Conclusion**

Both newly diagnosed CD patients and patients adhering to a GFD frequently demonstrate nutrient deficiencies which can have important clinical consequences. Nutrient deficiencies with known clinical relevance in CD patients include iron, vitamin D, folic acid, zinc and calcium at the time of diagnosis and iron, vitamin D, vitamin B6 and zinc during treatment with GFD.

In newly diagnosed CD patients these deficiencies may reflect the loss of absorptive surface area as well as functional capacity. Following the elimination of gluten from the diet, improvement in small intestinal histology occurs gradually, especially in adult patients. Consequently, recovery of nutrient deficiencies after diagnosis takes time. Nevertheless, even during long-term GFD treatment in CD patients with biopsy-proven remission, these patients may still show nutrient deficiencies due to insufficient nutrient intake.

Several known comorbidities, e.g. osteoporosis, anemia and neurological symptoms, are possible indicators of the impact that impaired nutritional status can have on CD patients. In pediatric CD patients, growth or impaired sexual maturation can be consequences that highlight the potential impact of impaired nutritional status in this patient group. Consequentially, assessment of nutritional status should be a part of both CD diagnosis and during follow-up. Additionally, dietary education for CD patients focusing not only on gluten elimination, but also the need to balance dietary patterns is important and should receive more attention.

Evidence on the benefit of nutrient supplementation on mucosal healing, correction of nutrient deficiencies or recovery from comorbidities associated with CD is inconclusive. While vitamin B12 and folic acid supplementation appear beneficial,

no evidence was found supporting favorable effects of calcium and vitamin D supplementation on decreased BMD in CD.

This review, although providing evidence for the relevance of nutrient status in CD at diagnosis and during treatment with a GFD, shows a noteworthy lack of high-quality evidence and a high degree of variability in the current literature regarding several relevant aspects. This concerns especially the lack of research in pediatric CD patients on a GFD and the consequences of impaired nutritional status on child development. The latter undoubtedly represents an important aspect in clinical care of these pediatric CD patients.

The causes of an impaired nutrient status in CD are currently only poorly understood. As improvement of nutritional status is of great importance for patients, further studies are warranted with comprehensive assessment of nutrient status in untreated and, even more importantly, treated CD patients are warranted. Studies should differentiate between adult and pediatric study populations and evaluate the need for optimal timing and dosing of supplementation as part of CD management. Prospective cohort studies would be expedient to further investigate the prevalence of certain nutrient deficiencies, their resulting health complications, and the potential role of nutrient supplements. It is of importance that future cohort studies are conducted on a multinational scale. This would not only account for differences between patient groups with different dietary habits, but also for patient groups that receive different types of medical care, from general practitioners and primary care facilities to specialized tertiary centers. Furthermore, researchers should pay more attention to the methodological aspects of assessing nutrient deficiencies, specifically regarding the type of measurement and appropriate reference values, clearly distinguishing between patients with sub-optimal nutrient levels and those with severe deficiencies. This will enable a more accurate evaluation of the scope of the problem and the clinical efficacy of treatment in the future. Long-term prospective studies could also provide evidence on the time it takes for nutritional deficiencies to recover and the factors influencing this process. This may shed light on the question whether we should strive to achieve a more rapid recovery of nutrient levels in CD patients after diagnosis. It could further clarify the matter if this can be achieved through attention to diet alone, or through addition of nutrient supplementation. In order to conduct valuable research addressing nutrient status in CD, it is crucial to reach a consensus on cut-off values of nutrient levels as well as the adequate techniques that should be used to assess them. When investigating the causes for the occurrence of certain deficiencies, the addition of a group of subjects (controls) following a GFD that do not have CD could be of additional value. This is currently more feasible, as GFD is becoming increasingly popular in healthy individuals without CD or in patients with non-celiac gluten sensitivity. This will yield important further knowledge to improve

overall management of CD. The goal of this should be to relieve symptoms, recover the intestinal mucosa, and reverse the consequences of CD-related malabsorption, while enabling patients to secure a nutritionally adequate GFD.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6643/12/2/500/s1>: Table S1: Overview of studies included in Tables 1 and 2, describing nutrient deficiencies in celiac disease patients with active and treated CD or nutrient intake in CD patients on a gluten-free diet.

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**Supplementary Material: Table S1**, Overview of studies included in Tables 1 and 2, describing nutrient deficiencies in celiac disease patients with active and treated CD or nutrient intake in CD patients on a gluten-free diet

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Tikkakoski et al. 2007	Prospective cohort study	Patients screened for CD	36 CD patients from 1900 adults screened for CD	Adults (18-64 years); in Finland	73%
Harper et al. 2007	Retrospective chart review	Adult CD patients Reference cohort: NHANES III	400	Mean age at diagnosis 46.5 years (SD 16.2); USA	67%
Vilppula et al. 2011	Prospective cohort study	Elderly population from the GOAL cohort	New CD patients: 35, screen detected from 2815 subjects	Median age at diagnosis: 61 (range 52-76); Finland	57%
Wierdsma et al. 2013	Cross-sectional single center cohort study 2005-2012	Consecutively diagnosed adult CD patients and healthy controls	CD: 80 Healthy controls: 24	(aged 18-75 years); Netherlands	65%
Zanini et al. 2013	Retrospective single center cohort study 1990-2010	Consecutively diagnosed CD patients	1408	Adult CD patients; Italy	73%
Schosler et al. 2016	Retrospective multicenter chart review 2008-2013	CD patients	93	>15 years ; one county in Denmark	76%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
Serum iron (median and number of patients with values below reference range of 9- 34 umol/l)	Test not described Reference ranges: 9-34 umol/l for S-Fe, 4.5-34 nmol/l for folate	2b
Hematologic blood values and biochemistry, Marsh grade	Not specified	2b
Celiac serology and HLA typing, clinical symptoms, BMD, fracture history, BMI, hematologic values and biochemistry	Not specified	3b
Marsh grade, BMI, biochemistry	Test not described, reference values provided: Vitamin A [1.2–3.0 nmol/L]; Vitamin B6 [13–80 nmol/L]; Folic acid [>5.6 nmol/L]; Vitamin B12 [150–700 pmol/L]; Zinc [11–19 nmol/L]; Vitamin (25-hydroxy) D [30–150 nmol/L]; Hemoglobin [M 8.5–11/F 7.5–10 mmol/L]; Ferritin [20–250 µG/L]	3b
Demographics, anthropometrics, clinical manifestations, biochemistry, hematology, celiac serology, BMD	Not specified	2b
GI symptoms, anthropometrics, biochemistry, and BMD	Test not described; Definition of abnormalities: Anemia: hemoglobin <57.3 mmol/l (women) and <58.3 mmol/l (men); Folate deficiencies: folate < 9 nmol/l; PTH elevation: parathyrin >6.9 pmol/l; vitamin B12 deficiency: cobalamin <200 pmol/l; iron deficiency: ferritin < 22 mg/l; vitamin D deficiency: 25-hydroxy-vitamin D2+D3 < 50 nmol/l; elevated alkaline phosphatase: alkaline phosphatase > 105mmol/l; low ionized calcium: < 1.18mmol/l.	2b

Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Sansotta <i>et al.</i> 2018	Single center retrospective chart review 2002-2015	Pediatric and adult CD patients	Children: 227 Adults: 327	Median (range) Children: 8.5 years (1.28–17.89) Adults: 38.9 years (18.3–75.7); USA	Children: 66% Adults: 78%
Berry <i>et al.</i> 2018	Single center prospective observational study 2012-2013	Consecutive patients, adult CD	103	Age at presentation (baseline) 26.15 ± 13.3 years; North India	53%
Haapalahti <i>et al.</i> 2005	Retrospective cohort study	CD (clinical diagnosis and through screening) and controls from cohort of healthy school children studying risk factors for type 1 Diabetes	CD patients: 26 Healthy controls: 29	16 to 25 years; Finland	69%
*Kempainen <i>et al.</i> 1998	Single center, Prospective intervention study on use of oats in CD 1988-1990	Adult CD patients, newly diagnosed	New CD: 40	Male: 47 ± 12 (24–65) Female ± 13 (18–62); Finland	70%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
GI and EI symptoms, prevalence of IDA	Not specified	2b
Clinical presentation, hematology, presence of anemia and folate, vitamin B12 and iron deficiency	Anemia: hemoglobin < 12 g/dL (females) and <13 g/dL (males) (WHO criteria); Iron deficiency: serum ferritin <30 ng/mL, serum iron <30 µg/dL, total iron binding capacity > 400 µg/dL, and percentage saturation < 15%; Folate deficiency: serum folate <4.0 ng/mL; vitamin B12 deficiency: serum B12 levels <200 pg/mL. Serum folate and B12 levels measured using a competitive immunoassay	1b
Biochemistry, celiac serology, BMI	Used reference values of Oulu University Hospital; serum ferritin <25 mg/L in females and <20 mg/L in males, whole blood folate <85 nmol/L, serum pre-albumin <0.23 g/L in females and <0.24 g/L in males and serum vitamin B12-vitamin <180 pmol/L were considered as low concentrations.	2b
Marsh, energy intake, reported symptoms, food records, anthropometrics and biochemistry	Methods measurement described for: Ferritin: 2b immunoluminometric assay; Vitamin B12: radioisotope dilution assay; Zinc: atomic-absorption spectrophotometry literature references for assays provided for vitamin D, E and A no reference values provided	

Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Lerner <i>et al.</i> 2011	Multi center cross sectional case control study	5 groups: New Pediatric and adult CD patients, Pediatric patients with nonspecific abdominal pain and their parents (adult control group)	Pediatric CD: 110 Adult CD: 22 Pediatric controls: 56 Adult controls: 84	Pediatric CD: 6±4 years (Israel) and 4±4 years (Spain) Pediatric controls 8±5 years Adult controls: 39±8 years (Israel) and 44±13 years (Spain); Israel and Spain	Children: 47 % Adult: 35%
Chakravarthi <i>et al.</i> 2012	Cross sectional cohort study 2008-2009	Adult new CD	54	Mean ± SD age of 30.6 ± 9.3 years (range 18-50); India	56 %
Garcia-Manzanares <i>et al.</i> 2012	Single center cross sectional study	Adult-onset consecutive CD patients	40	18 - 68 (mean 44.25 years); Spain	90%
Posthummus <i>et al.</i> 2017	Retrospective single center cross-sectional study	Adult-onset CD	283	Mean age at diagnosis 39 ± 12 (range: 18–67) years; Netherlands	78%
*Kempainen <i>et al.</i> 1999	Prospective study 5 year follow-up 1990-1991	Adult new CD	28	Age (years) Female: 44.1±13.6 (23.0-66.0) Male: 48.6±12.3 (24.0-65.0); Finland	68%
*McFarlane <i>et al.</i> 1995	Single center cross sectional cohort 1991-1992	Adult-onset CD	55	Men: 50.2 years (range 27.0-65.0 years) Women: 51.3 years (range 33.6-69.1 years); United Kingdom	82%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
Serum vitamin D Symptoms, familial disease, ethnicity, CD serology and biochemistry, histology	Measurement 25(OH) vitamin D serum concentration: commercial kit, LIAISON® 25(OH)D Assay (DiaSorin, Italy); Deficiency defined as <20 ng/ml according to Saintonge et al (2009)	3b
BMD, biochemistry, CD serology, Marsh classification	25 (OH) vitamin D levels were estimated by radioimmunoassay (normal values are >20 ng/mL).	3b
CD serology, HLA type, Marsh classification, biochemistry and urine, WHO fracture risk assessed by FRAX tool, BMD	Not specified	3b
Biochemistry, Marsh classification, BMD	Not specified	2b
BMI, BMD, medication and supplement use, four day food record, calcium, vitamin D and PTH	Serum 25-hydroxy vitamin D assay described by Törnquist and Lamberg-Allardt (1987) At 5 year follow-up: serum 25(OH)D concentration measured by commercial radio immunoassay (Incstar, Stillwater, MN) (blood drawn in autumn)	3b
Dietary assessment, BMI, biochemistry, BMD	Not specified	3b

Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Fernandez <i>et al.</i> 2010	Retrospective chart review 1990-2008	Adult-onset CD	68	Median age 36 years (range: 18-65); Spain	74%
*Hallert <i>et al.</i> 2002	Prospective multi-center cohort study 23% loss to follow up; compared to Nordic reference population and historic reference population	Adult CD 8-12 years of treatment with GFD; biopsy proven remission	30; Nordic reference population: 592	Mean age 55 years; 95% confidence interval (CI), 53.2-56.8; Sweden	60%
				Historic reference population: 504	
*Crofton <i>et al.</i> 1983	Intervention study evaluating zinc absorption	Adult CD, newly diagnosed and on a GFD; Healthy controls	CD: 22 Controls: 15	CD: Untreated: 47 (range 16-78) Treated: 38 (range 23-57) 35 (range 25-50); UK	CD: 68% Controls: 20%
*Boyd <i>et al.</i> 1985	Single center retrospective cohort study 1969-1983	Adult and Pediatric consecutive CD patients	50	Range 13 to 71 years (median, 30 years); UK	68%
Kuloglu <i>et al.</i> 2009	Retrospective single center chart review 1998-2006	Pediatric CD	109	Mean age 8.81 ± 4.63 years; Turkey	60%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
Biochemistry, homocysteine levels, C677T mutation in methylene-tetrahydrofolate reductase (MTHFR) gene	Not specified	3b
Biochemistry, plasma homocysteine, CD serology, 4-day food record, anthropometrics, Marsh classification, basal metabolic rate predicted using Schofield equations	Vitamin B-12: radioimmunoassay; plasma pyridoxal 5'-phosphate & total plasma homocysteine: enzymatic photometry with high-performance liquid chromatography separation (Mimelab AB, Söråker, Sweden) Reference range: Blood hemoglobin (g/L): 130–165 for males, 120–150 for females; Plasma ferritin (lg/L): 30–230 for males, 30–150 for females; Serum calcium (mmol/L): 2.2–2.6; Serum zinc (lmol/L): 11–17.7	2b
Plasma zinc concentrations, zinc tolerance test	Not specified	4
Presenting symptoms, anthropometrics, CD serology, test for malabsorption	Hemoglobin <12 g/dl; Folic acid <2.0 pg/l; Vitamin B12 <200 ng/l; Iron- men <16 pmol/l, women <11 pmol/l; calcium < 2.20 mmol/l; magnesium <0.70 mmol/l; zinc <8.4 pmol/l	4
Biochemistry, CD serology and autoantibodies, BMD, HLA genotype, BMI, anthropometrics,	Not specified	3b

## Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Wessels <i>et al.</i> 2016	Single center retrospective chart review; 2009-2014	Pediatric CD, newly diagnosed and on routine check up	182	Mean age at diagnosis 6.3 years ( $\pm 4.3$ ); Netherlands	65%
Deora <i>et al.</i> 2017	Single-center cohort study; at diagnosis, 6 and 18 months after starting a GFD, including patients receiving supplementation 2012-2016	Consecutive pediatric CD patients (<17 years)	140	Mean age 7.8 $\pm$ 4.01 years; Canada	62%
Zanchi <i>et al.</i> 2008	Prospective case-control study 2004-2005	Untreated pediatric CD patients and healthy children	CD: 54 Controls: 60	CD: mean age: 7.4 $\pm$ 4 years; range, 1.5 to 15 years Controls: mean age: 8 $\pm$ 3 years; range, 2 to 16 years; Italy	CD:59% Controls: 58%
Mager <i>et al.</i> 2012	Single center prospective cohort Diagnosis and 1 year follow up (23% loss to follow-up) 2009-2010	Pediatric CD patients	43	At diagnosis 9.4 $\pm$ 4.2 years (3 to 17); Canada	64.8%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
Biochemistry, Marsh grade, HLA genotype, CD serology	Ferritin, folate, vitamin B12: electrochemiluminescence immunoassay using Roche Modular E170; Roche Diagnostics, Basel, Switzerland); Calcium: Roche Modular P800; Roche Diagnostics); vitamin D: electrochemiluminescence immunoassay using Roche Modular E170; Roche Diagnostics) Reference values: Hemoglobin: age <7 y <6.9 mmol/L (<11.0 g/dL), age 7-15 y <6.5 mmol/L (<10.4 g/dL), age >15 y <6 mmol/L (<9.6 g/dL); Ferritin: age <5 y <12 ug/L, age ≥5 y <15 ug/L; Folate: <10 nmol/L (<4.45 ng/mL); Vitamin B12 <150 pmol/L (203 pg/mL); Calcium<2.15 mmol/L; Vitamin D (25[OH]D) <50 nmol/L (<20.8 ng/mL)	2b
Biochemistry, CD serology, marsh grade, dietetic assessment of GFD	Not specified	4
Biochemistry, CD serology, BMD, marsh grade	Calcium (normal values, 9.2 to 11 mg/dL), magnesium (1.80 to 2.30 mg/dL), 25(OH) vitamin D3 (20 to 120 ng/mL),	1b
BMD, marsh grade, anthropometrics, vitamin D/K status, diet, physical activity and sunlight exposure	Performed in the Core Laboratory at the UA Hospital according to standard methodologies; serum 25(OH)-vitamin D: deficient <50 nmol/l, suboptimal 50-75 nmol/l, sufficient >75 nmol/l according to Holick et al., 2007; Calcium (mmol/l) 2.1-2.6; Magnesium (mmol/l) 0.7-1.0 mmol/l	2b

Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Tokgöz <i>et al.</i> 2018	Single center case control 2015-2016	Consecutive Pediatric CD patients; healthy controls	CD: 52 Controls: 50	Range 0-18 years Median age CD: 9 ± 4.3 years for CD; Median age controls: 8.7 ± 5.2 years; Turkey	CD: 52% Controls: 50%
*Kavak <i>et al.</i> 2003	Case control study 2000-2001	Pediatric CD patients new and on a GFD; gender- and age matched healthy control subjects; None received supplements	Untreated CD: 34 CD on 1 year GFD: 28 Matched controls: 64	New CD: mean age 7.6 ± 4.7 years (range, 2-15 years); CD on GFD: mean 7.9 ± 4.7 years (range, 2-16 years); Turkey	New CD: 62% CD on GFD: 57% Controls: 47%
Tau <i>et al.</i> 2006	Longitudinal and prospective study 1995-2001	Pediatric CD consecutive patients received 1000 IU vitamin D per day during first 3 months of GFD; Compared to historic cohort of French children (Glastre <i>et al.</i> , 1990)	24	Mean age at diagnosis: 4.9±74.30 years (range 1-11.7 years); Argentina	75%
Volkan <i>et al.</i> 2018	Prospective case-control study 2015-2016	Pediatric CD; Age- and sex-matched healthy controls	New CD: 26 CD on GFD: 46 Controls: 30	Mean age CD: 11.69±3.04 Controls: 12.27±2.12; Turkey	Not specified
Rawal <i>et al.</i> 2010	Prospective RCT 2006-2007	Consecutive Pediatric CD patients with and without zinc supplementation	134	Mean age 6.71± 3.45 years (range 2-14 years); India	38%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
Vitamin D, K, E,A, CD serology, Marsh grade, anthropometrics, symptoms at diagnosis	Vitamin D: Chemiluminescence method in Architect hormone autoanalyzer (C8000 Architect, Abbott, Abbott Park, IL, USA). vitamin D insufficiency < 30 ng/ml, vitamin D deficiency < 20 ng/ml	2b
Biochemistry, BMD	Serum calcium: normal, 8.8–10.8 mg/dL; 25 -hydroxy vitamin D (25-OHD3): normal 9–45 ng/mL, determined with radioimmunoassay (RIA kit, Diasorin Inc., Stillwater, MN), intra-assay coefficient 6.1%, inter-assay coefficient 7.1%	4
BMD, biochemistry	Serum calcium atomic absorption spectrophotometry (normal value: 2.20–2.70 mmol/l); 25 hydroxy vitamin D in-house radiocompetitive protein-binding assay (Delvin et al., 1980) normal values: 92.5722.5 nmol/l in summer and 62.57 25 nmol/l in winter	3b
BMD, vitamin D, K, CD serology	Not specified	3b
Biochemistry, hematology, CD serology	Plasma zinc measurement: atomic absorption spectrophotometer (PERKLIN ELMER 400) Normal values (standardized in lab): 70–110 mgdL <sup>-1</sup>	1b

Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Rujner et al. 2004	Single center cohort study	Pediatric CD, new and on GFD Healthy controls	New CD: 28 On GFD: 41 Controls: 8	Female: mean age 13.04 years, range 5.92-18.3 Male: mean age 12.3 years, range 5.9-16.7; Poland	CD: 38%
Wild et al. 2010	Prospective single-center cohort study 2007-2008	Consecutive CD strict GFD for 6 months or more; 2 control populations: National Diet and Nutrition Survey of Adults (NDNS) Northern (n = 256) UK Women's Cohort Study (UKWCS) (n = 708)	93	Median age (years) Male: 63 (18-74) Female: 53 (21-79); UK	67%
Kinsey et al. 2008	Cross-sectional postal survey, CD diagnosis confirmed by gastroenterologist 2002, 2003	Adult CD Reference population: NDNS Henderson et al., 2002, 2003	49	Mean age 58.6 (±17) years; UK	76%
Shepherd et al. 2013	Prospective cohort study	New CD and, CD on a GFD	New CD: 50 CD on GFD: 55	Median age 44 (range 18-71) years; Australia	76%
Thompson et al. 2005	Cross sectional survey study	Adult CD patients	47	Mean age 51 years (SD:11) range 21-73 years; USA	83%
Mijatov et al. 2016	Prospective cohort study	Women with CD on strict GFD	40	Mean age in years 66.4 (range 41-109); Slovenia	100%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
Presenting symptoms, Mg retention, serum, erythrocyte, and urine Mg concentration, Mg dietary intake	Tissue magnesium deficiency: magnesium retention > 40% of intravenous load; Mg concentrations in serum, erythrocyte, and urine: flame atomic absorption spectrometry; Normal values Mg concentrations: Mg serum (mmol/l) (min-max) 0.7–1.04m, Mg erythrocyte (mmol/l) 1.8–2.4, Mg in urinary excretion (mmol/24 h/1.73m <sup>2</sup> ) >2.0	2b
Nutrient intake (prospective validated 5-day food diary), CD serology, comorbidities, reported dietary supplementation	EPIC validated food diary (Bingham et al. 2001)	2b
Three-day food diary, food questionnaire; Calculated mean daily intake of macro and micronutrients	Not specified	3b
Hematology, biochemistry, Marsh grade, seven-day prospective food record, structured interview: symptoms, demographics, anthropometry, previous dietary patterns, medical details	Detailed protocol, Measuring cups, spoons and reference diagrams provided	2b
Three-day estimated self-reported food records	Not applicable	4
Three-day food diary; Resting metabolic rate, physical activity level	Not applicable	3b

## Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
*Grehn et al. 2001	Cross sectional cohort study 1996-1997	Adult CD patients on GFD Reference population: Swedish national dietary survey in 1989, Becker et al.	49	45-64 years; Sweden	65%
*Collins et al. 1986	Single center cross sectional cohort	Adult CD patients	18	Mean age 44 years (range 18-59); UK	50%
Pham-Short et al. 2017	Case-control study	Pediatric CD and Type I diabetes	T1D+CD: 10 Type I diabetes only: 7	Children Type I diabetes+ CD: $14.3 \pm 3.6$ Type I diabetes only: $14.7 \pm 2.8$ ; Australia	-
Valente et al. 2015	Cross-sectional single-blind study 2011-2012	CD	CD: 20 Healthy controls: 39	Adults $36.3 \pm 13.7$ years; Brazil	65 %
*Bottaro et al. 1999	Multicenter (42 participating centers) retrospective cohort study 1990-1994	Adult and Pediatric consecutive patients with silent/ subclinical CD	1026 (644 children, 382 adults)	Mean age: Children: $7.7 \pm 4.2$ Adults: $24.4 \pm 12.5$ ; Italy	Children: 77% Adults: 71%

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
4-day food record, biochemistry	Blood samples analyzed according to local routines, except for zinc: atomic-absorption spectrophotometry	3b
Anthropometrics, symptoms, dietary history assessed by dietician	Not applicable	4
Continuous glucose monitoring; Standardized weighed food diary for three days	Food diaries analyzed using Foodworks 7 (Xyris, Australia), which uses Food Standards Australia New Zealand (FSANZ) published AusNut and NUTTAB 2010 databases	3b
3-day food records, serum concentrations of homocysteine, vitamin B6, B12, and folate determined after overnight fasting	Homocysteine (Immulite2000, Siemens, USA), 3b Roche, Switzerland) by chemiluminescence, vitamin B6 determined through analysis of PLP, isomer of highest concentration in human plasma, through High-Performance Liquid Chromatography with fluorescence detection Kimura et al 1996, Deitrick et al. 2001	
Medical history, comorbidities, demographics, symptoms, hematology, CD serology	Not specified	3b

## Supplementary Material: Table S1, Continued

Paper	Study design	Subjects	Number of subjects	Age and demo-graphics	% female
Öhlund et al. 2010	Baseline values of a prospective probiotics intervention study 2004	Pediatric CD patients on GFD	25	4-17 years; Sweden	72%

Overview of studies included in Tables 1 and 2 of the review reporting on nutrient deficiencies and dietary nutrient intake at diagnosis and on a gluten-free diet. \* Studies that did not meet the quality criteria were only included if no other eligible article existed for the nutrient and are marked by an asterisk. Evidence was assessed using the 2009 Oxford Centre for Evidence-Based Medicine Levels of evidence, were level of evidence could be graded down based on quality and if the study did not match the research question of this review.

Abbreviations: celiac disease (CD), gluten-free diet (GFD), Oxford Centre for Evidence-Based Medicine (OCEBM), serum iron (S-Fe), National Health and Nutrition Examination Survey(NHANES), United States of America (USA), Good Ageing in the Lahti region (GOAL cohort), human leukocyte antigen (HLA), bone mineral density (BMD), body mass index (BMI), Gastrointestinal symptoms (GI symptoms), parathyroid hormone (PTH), Extra intestinal symptoms (EI symptoms), iron deficiency anemia (IDA), world health organization (WHO), WHO fracture risk assessment tool (FRAX tool), United Kingdom (UK), Magnesium (Mg), National Diet and Nutrition Survey of Adults (NDNS) , UK Women's Cohort Study (UKWCS), European Prospective Investigation of Cancer study (EPIC study), C-reactive protein (CRP), Nordic Nutrition Recommendations (NNR)

Outcome parameters	Description of tests and reference values used for biochemistry	Level of evidence (OCEBM 2009)
5-day food records, anthropometrics,	Use of household measures for quantities; Individual energy requirement calculated according to Nordic Nutrition Recommendations 2004 (NNR-04)	3b



# NUTRIENT DEFICIENCIES IN CHILDREN WITH CELIAC DISEASE DURING LONG TERM FOLLOW-UP

Nutrient serum levels in pediatric patients with celiac disease following a gluten free diet

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\* equal contribution

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## **Abstract**

### **Background**

Celiac disease (CD) is associated with malabsorption and consequential nutritional deficiencies. Patients with CD must follow a gluten-free diet (GFD), which is also associated with nutrient deficiencies. Despite the clinical significance, consensus is lacking on the pattern and frequency of nutrient deficiencies in CD and the usefulness of assessment during follow-up. The aim was to investigate the presence of micronutrient and protein deficiencies in pediatric patients with CD after starting a GFD and receiving standard clinical care, taking disease activity into account.

### **Methods**

This single center retrospective chart review aimed to map the occurrence of nutrient deficiencies, determined in serum samples derived during follow-up in an expertise center for pediatric CD. Serological micronutrient levels were determined during routine clinical visits up until 10 years in children with CD on a GFD.

### **Results**

The data of 130 children with CD was included. When pooling the measurements between 3 months and 10 years after GFD initiation, a deficiency in iron, ferritin, vitamin D, vitamin B12, folate and zinc was present in 33%, 21,9%, 21,1%, 2,4%, 4,3% and 8,1% of measurements, respectively. No hypocalcemia or vitamin B6 deficiency was found.

### **Conclusion**

The prevalence of deficiency varies amongst nutrients in children following a GFD, a high prevalence of some nutrient deficiencies is noteworthy. This study highlights the necessity to structurally investigate the risk of developing nutrient deficiencies while following a GFD. Knowledge of the risk to develop deficiencies can contribute to achieving a more evidence-based approach in the management and follow-up of CD in children.

**Keywords:** Celiac disease; pediatric celiac disease; micronutrients; nutrients; follow-up; gluten-free diet

## 1. Introduction

The medical management of patients with celiac disease (CD) encompasses many challenges. Safeguarding nutritional status is one of them. CD is a chronic disease characterized by an auto-immune response elicited by gluten. The disease, that is often unnoticed long before diagnosis, can present itself during childhood but can also develop at an adult age. The prevalence in Europe is estimated to be between 1:70 to 1:300 [1, 2]. The treatment of CD consists of a strict gluten-free diet (GFD) in which grains of wheat, rye and barley should be avoided [3]. Currently, in Europe there are clear criteria for the diagnosis of CD in children [4] but there is no consensus with regards to testing and monitoring nutritional status during follow-up [5]. Insufficient studies were available investigating nutrient levels. They did not encompass the broad patient population and only investigated a limited number of different nutrients. Moreover, the data concerning nutrient status at moment of diagnosis and during short-term follow-up is already quite limited, whereas developments during long-term follow-up have barely been investigated. Furthermore, the extent of clinically relevant consequences on the short and long-term, are uninvestigated. A negative impact on growth and development later in life or the development of comorbidities such as impaired bone health due to (transient) deficiencies may be one of these consequences. Micronutrient deficiencies in active CD are thought to result from the incapacity of the damaged small intestine to absorb nutrients [6]. After initiation of a GFD, the small intestine will recover and regain its function, consequentially nutrient deficiencies are generally expected to recover as well. Maintaining a balanced diet that is gluten-free, can be a challenge which is why regular monitoring of CD mostly includes monitoring of blood micronutrient values. On the one hand it is important to avoid too much unnecessary testing. On the other hand, reduction of testing should not lead to missing nutrient deficiencies and their consequences. There is a large variation in reported frequency and types of deficiencies [7]. The inconclusive body of evidence is impeding the development of specific, evidence based guidelines on the monitoring of nutritional status in pediatric CD. Taken into account that pediatric patients with CD face lifelong follow-up under medical care, unambiguous and universal agreements regarding management are crucial. A universal policy that is scientifically founded, would help many patients with CD and their care providers and could prevent over- or under-diagnosis. This study focused on micronutrient status in the course of follow-up, as it occurs in patients with pediatric CD being diagnosed and followed up as part of standard clinical practice. The aim of this study was, to investigate the presence of micronutrient and protein deficiencies in pediatric patients with CD after starting a GFD, taking disease activity into account.

## **2. Materials and Methods**

In this single center retrospective chart review, data from 130 pediatric patients with CD, visiting Maastricht University Medical Centre (MUMC+) were included. The MUMC+ is a Dutch academic hospital and center of expertise for the care of patients with pediatric CD. All patients between 0 and 18 years of age receiving treatment in MUMC+ who were diagnosed with CD according to the ESPGHAN (European Society for Pediatric Gastroenterology, Hepatology and Nutrition) criteria were included[2, 4, 8]. Electronic patient files of pediatric patients who visited the hospital with a suspicion of CD between July 1998 and March 2021 were screened for inclusion.

Exclusion criteria were the presence of comorbidities that are likely to have an influence on micronutrient concentrations. Therefore patients with down syndrome, inflammatory bowel disease and patients with diabetes mellitus type 1 were excluded. Patients with thyroid autoimmune disorders were not excluded from this study. Patient information was retrieved from medical records and documented in the database at diagnosis, after  $3\pm 2$  months, after  $12\pm 5$  months and annually with a range of 5 months hereafter. Medical history, anthropometric measurements, physical examination, blood levels of CD antibodies, HLA genotyping and laboratory analysis were retrieved from the electronic patient charts as well. The CD antibodies measured consisted of IgA anti-tissue transglutaminase (IgA anti-TTG), IgA anti-endomysium (EMA) levels and total IgA levels. In case of IgA deficiency, IgG anti-tissue transglutaminase (IgG anti-TTG) was measured. The antibodies were used as an indicator for disease activity and are expected to be elevated at moment of diagnosis and when the patient does not adhere strictly to a GFD [9, 10].

The primary endpoints of the study are micronutrient and protein concentrations in the blood at diagnosis, after 3 months and annually up until 10 years after initiation of a GFD. The data analysis was executed in a coded fashion. The blood micronutrient levels examined were of calcium, iron, vitamin B6, vitamin B12, vitamin D, folate and zinc. Additionally, hemoglobin (Hb) levels were evaluated in combination with ferritin, to determine the presence of iron deficiency anemia (IDA). IDA was defined as the presence of ferritin and Hb levels below the threshold value. Blood samples were drawn as part of standard clinical practice. The majority of samples were analyzed in the MUMC+ laboratory. In some cases, patients had their blood drawn in other Dutch hospitals, where they were subsequently analyzed. The cut-off values of the MUMC+ hospital laboratory were used for all nutrient serum values (see supplementary table S1).

#### *Standard clinical care MUMC+*

When patients are diagnosed with CD, their medical history, current complaints and physical examination is documented. Besides this, a laboratory assessment is performed (see table 1). Approximately 3 months after initiation of a GFD and on an annual basis, a follow-up visit takes place where all of the measurements are repeated. Results of these measurements were used by the treating physicians for dietary advice and possible other necessary treatment, following standard clinical practice.

#### *Data analysis*

The frequency of mild and severe nutrient deficiency was calculated per nutrient, per follow-up moment until 10 years after initiation of a GFD. Frequencies of nutrient deficiencies were calculated for the entire patient group and subsequently compared to the frequencies in patients that showed normal IgA anti-TTG levels at the moment of follow-up measurement, using a Mann Whitney-U test. Normality of distribution of the data was tested using Statistical Package for the Social Sciences (IBM SPSS statistics version 25 for windows).

Ethical approval was obtained by an accredited medical research ethics committee namely the METC (in Dutch: medisch ethische toetsingscommissie) according to METC protocol 2019-2020.

### **3. Results**

Electronic patient files of 180 cases from July 1998 to march 2021 were investigated. Records from 144 patients were included for analysis, including 77 girls (59,2%), with a mean age at diagnosis of 6,2 years and a standard deviation of four years. Fourteen patients were excluded, because of comorbidities and 36 patients were excluded because they were not diagnosed with CD according to the European guidelines. Three patients included in the study were diagnosed with hypothyroidism, one of which had Hashimoto's thyroiditis. All three patients were treated with levothyroxine.

Three months after initiation of a GFD, IgA anti-TTG levels were available of 90 patients, 44 (49%) of which showed normal values below the threshold and 27 (34%) still having elevated anti-TTG levels. Twelve months after initiation of a GFD, 54 out of 81 (66%) patients had normal IgA anti-TTG values, while the remaining 17 patients still had elevated levels. Two and three years after initiation of a GFD respectively 75% and 77% of patients showed normal antibody levels.

**Table 1: Micronutrient and protein deficiencies in pediatric patients with celiac disease (CD) between 3 months and 10 years after initiation of the gluten-free diet (GFD).**

Micronutrient and marker of nutrient status	Number of patients with CD with deficiencies per follow-up moment after GFD initiation (%)									
	3 (+-2 months)	1 year (+-5 months)	2 years (+-5 months)	3 years (+-5 months)	4 years (+-5 months)	5 years (+-5 months)	6 years (+-5 months)	7 years (+-5 months)	8 years (+-5 months)	10 years (+-5 months)
Calcium	0/64 (0)	0/54 (0)	0/36 (0)	0/27 (0)	0/17 (0)	0/19 (0)	0/13 (0)	0/6 (0)	0/8 (0)	0/7 (0)
Ferritin	21/58 (36,2)	15/49 (30,6)	8/41 (19,5)	4/30 (13,3)	2/21 (9,5)	2/16 (12,5)	2/8 (25)	0/7 (0)	1/9 (11,1)	1/5 (20)
Iron	29/69 (42)	20/55 (36,3)	15/38 (39,4)	8/30 (26,6)	4/19 (21)	6/17 (35,3)	4/14 (28,5)	2/8 (25)	1/8 (12,5)	1/9 (11,1)
Hemoglobin	8/20 (40)	9/28 (32,1)	10/28 (35,7)	1/21 (4,8)	1/15 (6,6)	0/13 (0)	0/9 (0)	0/10 (0)	0/7 (0)	0/12 (0)
IDA	2/13 (15,4)	2/14 (14,2)	3/15 (20)	1/15 (6,7)	1/12 (8,3)	0/7 (0)	0/6 (0)	0/4 (0)	0/2 (0)	0/5 (0)
Vitamin B6	0/34 (0)	0/35 (0)	0/23 (0)	0/12 (0)	0/16 (0)	0/11 (0)	0/5 (0)	0/5 (0)	0/7 (0)	0/5 (0)
Vitamin B12	1/70 (1,4)	2/56 (3,5)	1/43 (2,3)	1/36 (2,7)	2/23 (8,6)	0/27 (0)	0/19 (0)	0/13 (0)	1/14 (7,1)	0/17 (0)
Folic acid	2/61 (3,3)	3/47 (6,3)	2/36 (5,5)	2/31 (6,4)	1/16 (6,2)	0/23 (0)	0/17 (0)	0/13 (0)	0/9 (0)	0/15 (0)
Vitamin D insufficiency	9/59 (15,2)	8/49 (16,3)	5/36 (3,8)	3/26 (11,5)	1/15 (6,6)	4/16 (25)	8/17 (45)	2/7 (28,5)	1/9 (11,1)	2/7 (28,5)
Vitamin D deficiency	2/59 (3,3)	1/49 (2)	2/36 (5,5)	0/26 (0)	0/15 (0)	0/16 (0)	1/17 (5,8)	0/7 (0)	0/9 (0)	0/7 (0)
Zinc	6/44 (13,6)	3/34 (8,8)	2/26 (7,6)	2/19 (10,5)	1/13 (7,6)	0/15 (0)	0/11 (0)	0/3 (0)	1/7 (0)	0/8 (0)
										0/6 (0)

The frequency of nutrient deficiencies per nutrient per follow-up moment up to 10 years was examined as well as the frequency of ferritin and Hb deficiency and IDA (see table 1).

During the 10 year follow-up period, a total of 256 calcium measurements were available. This included multiple repeated measurements of patients over the course of their follow-up. For vitamin B6, 176 measurement were available. For both nutrients, no deficiencies were measured at any time point (see table 1).

Ferritin was measured 265 times and a deficiency was seen 58 times. The frequency of a ferritin deficiency seemed to decrease over time, with 36% of patients having a deficiency 3 months after GFD initiation compared to 9,5% of patients after 5 years of follow-up. For iron a similar trend was seen, with 90 of a total of 273 measurements showing a deficiency. After following a GFD for three months, 42% of patients had a deficiency, compared to 35,3% 5 years after diagnosis (see table 1).

Three hundred thirty-one measurements were available of vitamin B12, eight of which were deficient. After following a GFD for three months and one year, 1,4% and 3,5% of patients had a deficiency respectively.

Twelve folic acid deficiencies out of total of 276 measurement were present. After initiation of a GFD, two out of 61 patients had a deficiency after three months and three patients after one year. One of the three patients with folic acid deficiency after one year was diagnosed with Hashimoto's thyroiditis which was treated at the moment of measurement.

Vitamin D was measured 247 times. Insufficiencies of vitamin D were present 46 times and deficiencies 6 times. Insufficiencies and deficiencies seemed to arise randomly with no specific trend over time. 15% of patients had insufficient vitamin D levels three months after initiation of a GFD and 16,3% of patients after one year. A deficiency was found in 3,3% and 2% of patients after three months and one year of following a GFD respectively (see table 1).

Zinc was measured 186 times and a deficiency was detected in 15 cases. Six out of 44 (13,6%) patients had a deficiency three months after initiation of a GFD, and three out of 34 (8,8%) had a deficiency one year after initiation of a GFD.

The detected nutrient deficiencies included cases of patients that showed deficiency at moment of diagnosis, which then persisted. However, in a substantial amount of the cases, the deficiencies arose newly. In case of ferritin deficiency that was measured during follow-up, 21% of the patients had known normal values prior to occurrence of the deficiency. This was the case in 24% of patients with iron

deficiency, 38% with vitamin B12 deficiency, 30% with vitamin D deficiency, 27% of zinc deficiency and 50% of cases of folate deficiency.

Frequencies of nutrient deficiencies in the total group were compared to the nutrient deficiencies in patients with proven normal IgA anti-TTG values (see table 2). During follow-up, the majority of patients showed normal antibody values, with 73 patients showing a normalization of celiac antibodies within one year after diagnosis. The occurrence of deficiencies did not seem to differ between the complete group of patients and the subgroup of children with measured normal antibody levels (see table 2). For instance, after one year of following a GFD, iron deficiency was measured in 45% of patients that showed normalized antibody levels, compared to 36% in the complete group.

**Table 2:** Micronutrient and protein deficiencies in pediatric patients with celiac disease (CD) one and two years after initiation of the gluten- free diet (GFD). The complete group with all available measurements is shown. Compared with the subgroup of patients in whom IgA anti-TTG (CD antibody) levels were available at the time of nutrient measurement and confirmed to be normal.

	Number of patients with CD with deficiencies one year after GFD initiation (%)		Number of patients with CD with deficiencies two years after GFD initiation (%)	
	Subgroup with normalized CD antibodies (<10)	Complete group	Subgroup with normalized CD antibodies (<10)	Complete group
Calcium	0/36 (0)	0/54 (0)	0/22 (0)	0/36(0)
Ferritin	9/33 (27,3)	15/49 (30,6)	6/24 (25)	8/41 (19,5)
Iron	17/38 (44,7)	20/55 (36,3)	11/22 (50)	15/38 (39,4)
Vitamin B6	0/25 (0)	0/35 (0)	0/14 (0)	0/23 (0)
Vitamin B12	2/36 (5,6)	2/56 (3,5)	1/27 (3,7)	1/43 (2,3)
Folic acid	2/31 (6,5)	3/47 (6,3)	2/20 (10)	2/36 (5,5)
Vitamin D insufficiency	5/34 (14,7)	8/49 (16,3)	2/20 (10)	5/36 (3,8)
Vitamin D deficiency	0/34 (0)	1/49 (2)	1/20 (5)	2/36 (5,5)
Zinc	2/25 (8)	3/34 (8,8)	2/15 (13,3)	2/26 (7,6)

## **4. Discussion**

In this retrospective chart review, a wide variety of nutrient deficiencies were observed in pediatric patients with CD following a GFD during the course of 10 years follow-up. This included deficiencies that persisted after diagnosis and also newly developed deficiencies, which highlight that nutrient status should receive

the attention of patients and professionals as part of CD management. The variance in deficiency between nutrients and markers monitored, was quite pronounced, with deficiencies in serum vitamin D, iron and ferritin levels occurring frequently and deficiencies in vitamin B12, folic acid and zinc occurring moderately frequent, while neither hypocalcemia nor vitamin B6 deficiency was found. Literature on distribution and prevalence of deficiencies in pediatric patients with CD shows similar distributions, albeit data is quite scarce. When comparing the results of different studies, it must be noted, that cut-off values as well as laboratory tests to determine serum nutrient values differ greatly, impeding comparability.

During 10 years of follow-up, both ferritin and iron deficiencies were the most prevalent in this cohort, with average frequencies of 21,9% and 33%, respectively and a decreasing prevalence over time. Other studies in comparable patient populations have shown similar results. A study in 26 patients with CD aged 16 - 25 years, showed a ferritin deficiency rate of 28% while following a GFD [11]. In a Dutch cohort of pediatric patients with CD conducted by Wessels *et al.* ferritin deficiency was found in 28% of children following a GFD [12]. Despite the differences in cut-off values among these studies, the current study appears to confirm that iron and ferritin deficiency is highly prevalent in children with CD following a GFD [13]. In comparison with the general pediatric population, this is a markedly higher rate. With an iron deficiency prevalence of 8,2% and 7,7% reported respectively in a Norwegian and Spanish child cohort (ages 1-12)) [14, 15].

In our patient cohort, vitamin D deficiency was present in 2,5% and insufficiency in 18,6% of measurements. Evidence seems to support this high observed prevalence. For children with CD, a study in two subgroups, one Israeli and one Spanish, had deficiency rate of 33,3% and 16,9% respectively [16]. Wessels *et al.* reported a frequency in vitamin D deficiency of 12- 25% during follow-up [12]. In contrast, Zanchi *et al.* found no cases of vitamin D deficiency in pediatric patients with CD after a mean of six months following a GFD. However, the study did not include follow-up measurements thereafter [17]. In a recent study Lionetta *et al.* found that vitamin D deficiency was more frequent in children with CD compared to healthy controls [18].

In contrast to these frequently occurring deficiencies, no cases of hypocalcemia or vitamin B6 deficiency were found during follow-up. Other studies in Dutch and Turkish pediatric cohorts also report no cases of hypocalcemia during follow-up [12, 19]. To our knowledge, no recent data exists on the vitamin B6 status in pediatric patients with CD following a GFD. Vitamin B6 deficiency has been described in adult patients with CD on a long-term GFD. However, vitamin B6 deficiency appears to be more prevalent in adults in general and is therefore not extrapolatable to the pediatric population [20, 21].

Vitamin B12, zinc and folate deficiencies were observed in this cohort during the entire course of the 10 year follow-up period, albeit markedly less frequent than iron deficiency. The body of evidence of the prevalence of these deficiencies in pediatric patients with CD is generally in line with these findings, although somewhat inconclusive.

The number of patients with a vitamin B12 deficiency in this cohort remained similar during the course of follow-up and was on average 2,4%. This is notably higher, compared to the reported prevalence in the general pediatric population. The United States National Health and Nutrition Examination Survey (NHANES) III study identified vitamin B12 deficiency in 0,5% of 3766 children aged 4-19 [22]. In contrast to the results of our study, Wessels *et al.* only reported one patient with vitamin B12 levels below threshold, after one and two years of follow-up, out of 73 and 71 patients respectively [12].

In the presented cohort, zinc deficiency occurred in 8% of the measurements. It could arise at any given time period and recovered in the next follow-up moment. Two separate studies found a high zinc deficiency rate of 71,6% ( $<10,7 \mu\text{mol/l}$ ) in 134 and 109 children with CD in India and Turkey respectively [23, 24]. This in contrast to a single center cohort study in 140 Canadian children which found a lower prevalence (18,6%) [25].

On average, a folate deficiency was detected in 4,3 % of measurements in this cohort and all deficiencies were newly occurring during follow-up. Wessels *et al.* found a deficiency in 0-3% ( $<10\text{nmol/l}$ ) of pediatric patients with CD during follow-up [12]. Data from the NHANES 1999-2000 showed 0,5% of children in the general population to have a folate deficiency ( $< 6,8 \text{ nmol/L}$ ) [26]. Although folate deficiency appears to occur in low numbers in patients with CD, this is higher than in the general population of children [26]. Occurrence of folate deficiency during follow-up appears to be unpredictable based on baseline measurements at moment of diagnosis.

There are three important reasons for nutrient deficiencies in patients with CD. First, the characteristic intestinal damage, can lead to impaired intestinal function and therefore reduced absorptive capacity [27]. During follow-up, the intestinal wall is expected to recover, although recovery duration can vary, and unintentional or intentional gluten ingestion during follow-up could play a role herein. CD antibodies can be used as an indicator for disease activity, although several studies have shown that their properties as a biomarker herein are not ideal [28]. In this cohort, no significant differences were observed between the prevalence of nutrient deficiencies in the total group, compared to the group of

patients with normal IgA anti-TTG values. Additionally, the self-reported dietary compliance in the cohort was good.

Second, the occurrence of the examined nutrient deficiencies in the general pediatric population must be considered. Factors independent of CD, can influence this, such as geographical location and local policies concerning (healthy) eating and supplementation. For example, a study examining vitamin D deficiency among healthy Canadian children, reported higher values of insufficiency (43%) than in the present study [29]. A contributing factor could be varying policies regarding vitamin D intake in children. For instance, it is a Dutch national policy that 400IE vitamin D are supplemented each day from birth until 4 years [30, 31]. It is however unclear, whether (temporary) deficiencies of micronutrients at diagnosis and during follow-up have different consequences in patients with CD compared to healthy controls. Vitamin D deficiency could be of greater importance in this patient population with a known increased risk for osteopenia and bone fractures later in life [32-34]. As this is unclear, the occurrence of nutrient deficiencies should be evaluated separately in this patient population.

Lastly, following a balanced, well-rounded GFD can be a challenge for patients and their parents [13]. The occurrence of new nutrient deficiencies in this cohort of patients under care in a tertiary center for pediatric CD, even after a long period of following a GFD, underlines the importance of a balanced GFD. For instance, a major dietary source of iron and B vitamins are gluten-containing grains, which must be replaced with products with inferior nutritional value [35, 36]. Patients with CD often fail to achieve the recommended daily intake of iron, which could explain the prevalence of iron deficiency and consequentially IDA in this cohort [37].

This should not only receive attention at moment of diagnosis, but counseling herein appears to stay necessary, even during long-term follow-up. Monitoring of serum nutrient values in pediatric patients with CD could also play a role in this counseling. Results can be used, not only in detecting deficiencies, but rather in preventing their occurrence. Declining nutrient values over time can be utilized as a signal for practitioners and patients, to identify possible shortcomings in the diet and counsel patients accordingly.

At this point the body of evidence is not sufficient to create sound, evidence based guidelines on monitoring nutrient status in pediatric patients with CD. This is reflected in the limited recommendations in the guidelines currently used in the Netherlands concerning this issue(see supplementary table S2 for an overview). More data is needed in order to make decisions on frequency of evaluation per nutrient, as well as the best way to assess their status in the body.. As frequency

varies greatly, it could be considered to categorize nutrients in order of prevalence of their deficiency, and plan monitoring accordingly. Further, future research should examine possible clinical consequences of (transient) nutrient deficiencies in pediatric patients with CD, in order to further assess their relevance. A less frequently occurring deficiency could have considerable impact on growth and development, warranting its continuous follow-up, regardless of prevalence [13]. For example, impaired bone health is an important comorbidity associated with nutrient deficiencies specifically in patients with CD [38, 39]. An increased fracture risk as well as increased risk for osteoporosis later in life have been associated with calcium and vitamin D deficiencies in CD [34, 40]. Strikingly, hypocalcemia has not been detected in this cohort, which is in line with the current literature. Measuring serum calcium concentrations could be an inadequate marker for bone health and calcium status in the entire body. This is illustrated by a study of Kuloglu *et al.* in which a prevalence of low bone mineral density (BMD) was found in 53,8% of cases compared to hypocalcemia in 0,9% of cases [24]. In line with this, studies show poor performance of biochemical parameters and superiority of bone densitometry [41][42]. Thus, vitamin D and calcium blood levels are poor indicators for bone health assessment and other methods to evaluate BMD should be considered. When drawing up guidelines specifically on the follow-up of nutrient status in patients with CD, not only frequency, but also mode of monitoring should be critically evaluated.

The risk of developing nutrient deficiencies and their possible consequential comorbidities should be considered along with, the burden of blood evaluations in pediatric patients. This entails the potential burden on the patients, as well as the financial burden on the health care system of possibly unnecessary testing, underscoring the need to critically evaluate the risks per individual nutrient.

#### *Strengths and limitations*

This study entails a relatively large group of pediatric patients with CD with a very long period of follow-up measurements, including the assessment of an abundant range of nutrients. Further, the cohort appears to approximate the general population of pediatric patients with CD, for example concerning sex distribution and age of diagnosis [43-45]. One of the main weaknesses is the sometimes limited availability of measurements per nutrient, due to the observational character of the study. A finding that is normal in a retrospective chart review but influences the strengths of the outcomes. Further, the study describes frequency of nutrient deficiencies in this population in general. Future research should incorporate additional information, such as supplement use of the patients and the potential health complications of nutrient deficiencies in order to provide more insight. The addition of a healthy control group would further deepen the understanding of the potential risk factors for nutrient deficiencies during follow-up.

## **5. Conclusion**

Children with CD not only show nutrient deficiencies at diagnosis, but also during follow-up while following a GFD. The frequency of occurrence varies greatly herein, with iron and vitamin D deficiencies occurring frequently in contrast to vitamin B6 and calcium deficiencies not being present at all. This highlights the necessity of good dietary guidance, not only focusing on gluten elimination, but also on nutritional value of a GFD, throughout the childhood of these patients. Comprehensive knowledge on prevalence of nutrient deficiencies in CD, including risk factors for their development and possible severity of their clinical consequences are necessary in order to create guidelines on the monitoring of nutritional status in pediatric patients with CD. This knowledge should help to make decisions on frequency, type of nutrient and form of monitoring in order to lower patient and financial burden on the one hand and prevent clinical consequences of nutrient imbalances on the other hand.

**Supplementary Materials:** The following are available online

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**Supplementary Table S1.** Cut-off points for micronutrient measurements in blood obtained by venipuncture in MUMC+ by gender and by age. **Supplementary Table S2.** Guidelines and their recommendations regarding follow up in children with celiac disease (CD).

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**Supplementary Table S1:** Cut-off points for micronutrient and protein measurements in blood obtained by venipuncture in MUMC+ by gender and by age.

Micronutrient and proteins	Age	Girls	Boys	Unit
Total calcium[46]	0-1 year	2.00 - 3.00		mmol/l
	1-12 years	2.10 - 2.90		
	>12 years	2.10 - 2.55		
Ferritin[46]		15-200	30-400	µg/l
<i>Mild ferritin deficiency</i>		8-15	15-30	
<i>Severe ferritin deficiency</i>		<8	<15	
Iron[46]		11.0 - 25.0	14.0 - 27.0	µmol/l
Hemoglobin (Hb)[46]	1-6 months	6.0 - 9.0		mmol/l
	0.5-2 years	6.8 - 8.1		
	2-8 years	7.5 - 8.7		
	8-15 years	7.1 - 9.0		
	>15 years	7.3 - 9.7	8.2 - 11.0	
Vitamin B6[46]		50 - 200		nmol/l
Vitamin B12[46]		145 - 569		pmol/l
Vitamin 25(OH)D3 [46]	0-18 years	deficiency <30 insufficiency 30-50		nmol/l
Folate[46]		8.0 - 60.8		nmol/l
Zinc[46]		9.3 - 15.1		µmol/l

**Supplementary Table S2:** Guidelines and their recommendations regarding follow up in children with celiac disease (CD).

Guideline	Make recommendation	Specific nutrients to follow-up	Interval
2012 ESPGHAN Guidelines[2]	Yes	No	'regularly'
2020 ESPGHAN guidelines[4]	No	-	-
Dutch guideline 'Celiac disease and dermatitis herpetiformis'[47]	Yes	At diagnosis anti-TTG or EMA, Hb, mean corpuscular volume (MCV), iron, vitamin status, thyroid stimulating hormone (TSH) and free thyroxine (FT4) Yearly Hb, hematocrit, folate, vitamin B12, calcium, AF, iron status, anti-TTG, TSH and FT4 on indication.	At diagnosis and yearly
Dutch society for CD[3]	Yes	Anti-TTG, Hb, mcv, iron status. TSH and FT4 on indication.	Yearly
Guideline 'European society for the study of celiac disease' [48]	Yes	Hb, hematocrit, leukocytes, thrombocytes, erythrocytes, MCV, MCH and MCHC, iron status, folate, vitamin B12, thyroid function, liver enzymes, calcium, phosphate, vitamin D	At diagnosis and 12 months after diagnosis. Liver enzymes yearly, the remainder only on indication of if abnormalities.

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# CELIAC DISEASE AND THE GLUTEN FREE DIET DURING THE COVID-19 PANDEMIC: EXPERIENCES OF CHILDREN AND PARENTS

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## **Abstract:**

The COVID-19 pandemic perturbed everyday life of children and those with a chronic illness and their families in particular. Patients with celiac disease (CD) follow a strict glu-ten-free diet (GFD) and gluten ingestion is associated with negative health outcomes. The aim of this study was to investigate the experiences of children with CD and their families during the first period of the COVID-19 pandemic concerning their GFD, symptoms and CD management. A cross-sectional questionnaire based study was performed, including thirty-seven Dutch pediatric patients with CD and their parents. The majority reported good compliance to the GFD and found that the diet was easier to follow during the pandemic, mainly due to eating more in the home. Some discovered a greater variety of GF products utilizing online shopping, potentially increasing the financial burden of the GFD. Concerning general dietary habits, 21,6% reported a healthier eating pattern, in contrast to 37,8% and 10,8% who consumed more unhealthy snacks and less fruit and vegetables, respectively, during the pandemic than normal.

The natural experiment of the COVID-19 pandemic provides valuable information on management of pediatric CD. Education on healthy dietary patterns is important, especially in children with restrictive diets, and the findings of this study show that there is room for improvement in this respect, regardless of the current pandemic.

**Keywords:** Celiac Disease; COVID-19; Gluten Free diet; Dietary Patterns; Disease Management; Patient experience

## 1. Introduction

The SARS-CoV-2 (COVID-19) outbreak and the national mitigation measures have temporarily led to a radical change in daily life. The infection itself, as well as the effects of the pandemic, could possibly have an even stronger impact on patients with chronic illness, along with their families. Celiac disease (CD) is a common chronic autoimmune disease that often manifests in childhood. The prevalence in the Netherlands is estimated to be 1%. Currently, a strict gluten-free diet (GFD) is the only treatment, which typically leads to complete symptom resolution. Gluten ingestion can have a number of adverse effects in patients with CD, including a flare-up of the auto immune reaction, with associated complaints and negative short and long term consequences. An additional challenge for patients with CD is ensuring a diet that is not only gluten-free, but also of high nutritional quality. Several government-issued rules and regulations were put in place in the Netherlands during the COVID-19 pandemic. These measures were introduced step-wise from March 2020 onward [1-3]. Consequently, altered behavior in everyday life could be observed, such as hoarding of groceries, as well as remote work and education. Dutch citizens were encouraged to practice social distancing by staying at home as much as possible. Sport clubs, restaurants, museums, and theaters, as well as schools and daycare centers, were closed [4]. Altered grocery shopping behavior led to shortages of common supermarket items such as flour, bread, and soap [5]. A shortage of certain food items, as well as limited access to food outside the home, might have affected eating habits and dietary patterns [6-8]. Initial investigations suggested that dietary patterns in the general population were unfavorably influenced by the lockdown period [9]. This was also found in a Dutch child cohort of the COLC study investigating the lifestyle and well-being of children since the beginning of the COVID-19 pandemic. The COLC study included 189 children in the Netherlands between the ages of 4 and 18 years. A subgroup of families participating in a qualitative study via semi structured interviews showed an overall unhealthier lifestyle and a decline in well-being since the start of the pandemic [10]. Additional analysis of quantitative data from the COLC cohort confirmed these findings (unpublished data from the COLC study: ClinicalTrials.gov Identifier: NCT04411511, accessed on 19 February 2023). It is unclear whether diet was also unfavorably impacted in children with CD due to the lockdown. Further, experiences with the GFD during the pandemic concerning issues such as gluten avoidance or product availability have not been examined. Due to the COVID-19 pandemic, medical care was drastically reduced, leading to postponement of hospital visits or diagnostic procedures [11]. Among other societies, the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition advised the postponing of medical care of patients with CD when possible, noting that this could lead to anxiety or other adverse outcomes in patients [12]. Consequentially, telehealth options were made available on a larger

scale, which appears to be a potentially positive option for this patient population [13–15]. Apart from expert opinions, there is limited empirical data available on the impact of the pandemic and its protective measures on children with underlying diseases and specific dietary restrictions. The primary goal of this study was to investigate the experiences of children with CD and their families during the COVID-19 pandemic in regards to adherence to, and quality of, the (gluten-free) diet, along with access to and availability of gluten-free products. Secondly, the impact of the national measures instituted by the Dutch government, as well as possible changes in societal behavior, on patients with CD was investigated.

## **2. Materials and Methods**

The study consisted of a cross-sectional questionnaire sent out to children with CD and their families during the lock-down measures of the COVID-19 pandemic in the Netherlands. Questionnaires were sent out at the end of June 2020. Responses were included until the beginning of September 2020. The study population included patients, 0–18 years old, of the Maastricht University Medical Centre (MUMC+) who were diagnosed with CD according to the ESPGHAN (European Society for Pediatric Gastroenterology, Hepatology, and Nutrition) guidelines [16]. Patients that were not diagnosed according to the ESPGHAN guidelines were excluded. After providing informed consent, parents of children under the age of 12 years, or the children themselves, if they were aged 12 years and older, completed the questionnaires at home. The questionnaire included baseline questions regarding the period prior to the COVID-19 pandemic, starting in February 2020 in the Netherlands, and questions concerning the period in which several measures were taken to limit the spread of the virus (for the translated questionnaires, see Supplementary Material S1). The questionnaire included five main domains. The first domain involved questions about symptoms related to CD activity or COVID-19 infection (see Supplementary Table S1). Patients reported on symptoms related to either CD activity, a possible infection with COVID-19, or both (concordant symptoms, such as diarrhea). The second domain was comprised of questions regarding the management of the GFD in general, as well as during the pandemic. The third domain contained questions on dietary habits in general, prior to and during the pandemic. In the fourth domain, patients and their parents were asked to what extend they followed the government-issued measures as a response to the pandemic. The fifth domain contained questions regarding perceived obstacles or possible benefits concerning CD, its management, or the CD-related care patients received during the pandemic. The questionnaire comprised multiple choice questions and open questions, with space for free text responses. The results were mainly descriptive and are presented as such in this manuscript. Statistical analysis was executed using SPSS version 25 (SPSS incorporated, Chicago, IL, USA). The

local institutional review board Medisch Ethische Toetsingscommissie (METC) of the Maastricht UMC+ approved the study, which was performed based on the Declaration of Helsinki. During the pandemic, the board of directors of Maastricht UMC+ adopted a policy to inform patients and ask their consent for COVID-19 research purposes.

### **3. Results**

A digital questionnaire was sent to 104 pediatric patients with CD and an identical paper version was sent by regular mail. In total, 36% (37 children) responded with a completed questionnaire, 67.6% of whom were female (n = 25). The mean age was 10.4 years (range 3 to 18 years). The median duration of diagnosis prior to participation was 52.5 months (ranging from 6 months to 16 years). There were seven patients who had followed a GFD for less than two years, while the others had received a more long-term diagnoses.

Patients answered 11 questions regarding their management of the GFD before the COVID-19 pandemic (see Supplementary Material S1). All 37 patients reported following the GFD strictly and taking measures to prevent unintentional gluten ingestion, such as discussing the diet when eating out of the home, storing gluten-free products separately from gluten containing food items, and only using gluten-free medicine products. A total of 94.6% (n = 35) of the children reported not eating products containing wheat starch, 64.9% (n = 24) of the children did not eat food labeled 'prepared in an environment where gluten is processed,' and half of the children (n = 19) did not eat food labeled 'may contain traces of gluten or wheat'. When asked about their experiences with the GFD during the COVID-19 pandemic, 6 patients (16.2%) stated that they ingested gluten during this period, 1 of which did so intentionally, whereas the other 5 patients reported unintentional gluten ingestion (see Table 1). The majority of the patients, (n = 31; 83.7%) were either certain or quite sure that they did not ingest gluten during this period.

**Table 1:** Reported consumption of gluten since start of the COVID-19 pandemic of children with celiac disease.

<b>Patients with celiac disease</b>	<b>Number of patients in percent (n)</b>
Intentional gluten ingestion	2.7% (n=1)
Unintentional gluten ingestion	13.5% (n=5)
Certainly did not ingest gluten	37.8% (n=14)
Think they did not ingest gluten	45.9% (n=17)

Patients were asked whether they developed new symptoms or experienced an increase in symptoms which could be related to either a COVID-19 infection, CD activity, or both (see Table 2; for the list of symptoms, see Supplementary Table S1). A total of 57% (n = 21) of the children reported new complaints or an increase in existing complaints during the COVID-19 pandemic (see Table 2). The common cold (n = 9), coughing (n = 6), fever (n = 5), sneezing (n = 6), and fatigue (n = 5) were the most prevalent symptoms among these patients. Only two patients underwent a nose and throat swab for PCR testing to determine whether an active COVID-19 infection was present, both with negative results. A total of 14 patients reported that one or more of their family members developed complaints that could be attributed to COVID-19. For this reason, 9 family members underwent a nose and throat swab, only 1 of which was positive for the mother of a patient. At the time the questionnaire was sent out (from June 1st onward), anyone with mild symptoms could be tested through the municipal health services in the Netherlands. Before 1 June 2020, testing was more limited due to limited resources [3].

**Table 2:** Percentage of pediatric patients with celiac disease (CD) that reported an increase or a new occurrence of symptoms either related to CD activity, a possible COVID-19 infection, or both (concordant symptoms) during the COVID-19 pandemic.

Patients with celiac disease	Percentage of patients (n)
Developed new complaints*	56.7% (n=21)
COVID-19 related symptoms	28.6% (n=6)
CD related symptoms	2.7% (n=1)
Concordant symptoms (symptoms related to CD activity and/or a possible COVID-19 infection)	66% (n=14)

\*For list of CD, COVID-19 and concordant symptoms, see supplementary table S2.

Concerning the availability of GF products during the COVID-19 pandemic, 67.6% of patients stated that there were enough products present at all times; 21.6% stated that they could not purchase the gluten-free products they would usually buy, but that enough gluten-free alternatives were available to maintain and ensure a GFD; and 10.8% reported a scarcity in gluten-free products during the pandemic. In response to the question regarding whether patients took specific precautions during the COVID-19 pandemic to ensure that their GFD was not comprised, 54.1% stated that they did indeed do so. These measures consisted of hoarding gluten-free products, ordering online groceries, and having products shipped from abroad. Patients that started buying gluten-free products online stated that as a side-effect, they discovered a new array of products they did not know about before, and which they would not have discovered if not for the COVID-19 pandemic.

Interestingly, 21.6% of the participants reported that they started eating healthier during the pandemic than during a normal school week (21.6%) (See Table 3). Healthy snacks were eaten more often in 29.7% of patients and 18.9% of patients stated that they ate more fruit and vegetables compared to the amount consumed during a normal school week. Patients also reported that buying groceries online was easier than going out to a store to buy gluten-free products (10.8%). Further, staying at home made it easier to adhere to the GFD, as reported by 32.4% of patients. One parent stated: “because we were at home more often, the eating environment was ‘clean’ which led to less contamination with gluten.”

**Table 3:** Experiences in eating pattern / perceived advantages and barriers regarding the gluten free diet (GFD) during the COVID-19 pandemic.

Advantages and Barriers	Percentages of patients (n)
<b>Experiences disadvantages</b>	
Eating pattern is unhealthier during the pandemic than during a normal school week	35.1% (n = 13) 18.9% (n = 7)
Less availability of products	29.7% (n = 11)
Ate unhealthy snacks more often	37.8% (n = 14)
Ate fruits and vegetables less often	10.8% (n = 4)
<b>Experienced advantages</b>	
Eating pattern is healthier during the pandemic than during a normal school week.	55.8% (n=19)
Doing groceries online makes it easier to purchase gluten free products	21.6% (n = 8) 10.8% (n = 4)
Staying at home more, makes the GFD easier to follow	32.4% (n = 12)
Ate healthy snacks more often	29.7% (n = 11)
Ate fruits and vegetables more often	18.9% (n = 7)

A significant ( $p = 0.01$ ) increase in the number of times patients or their parents cooked at home was also observed (see Table 4). No significant differences were observed in other dietary habits, such as the number of fruits and vegetables eaten daily, water and soda intake, snacking behavior, or ordering take-away. A total of 16 out of 37 children achieved the daily norm for fruit intake of 1.5 pieces of fruit per day before the COVID-19 pandemic, and 17 children achieved this norm during COVID-19.

**Table 4:** Reported eating behavior of children with celiac disease (CD) before and during the COVID-19 pandemic.

Eating behavior during COVID-19 pandemic compared to behavior prior to the pandemic	Percentage of children that reported a change in eating behavior during the COVID-19 pandemic compared to before	P value of difference in reported number during as compared to prior to the COVID-19 pandemic
<b>Fruit intake</b>		
Achieved the norm before COVID-19	43.2% (16/37)	
Achieved the norm during COVID-19	45.9% (17/37)	
<b>Eating fruit</b>		
Ate less fruit during COVID-19	19.4% (7/36)	
Ate more fruit during COVID-19	8.3% (3/36)	0.928
<b>Drinking water</b>		
Drank less water during COVID-19	10.8% (4/37)	
Drank more water during COVID-19	5.4% (2/37)	0.739
<b>Drinking soda</b>		
Drank less soda during COVID-19	2.7% (1/37)	
Drank more soda during COVID-19	8.1% (3/37)	0.705
<b>Cooking at home</b>		
Cooked at home more often during COVID-19	27% (10/37)*	
Cooked at home less often during COVID-19	0% (0/37)	0.002*

\* Significant difference between reported number during the COVID-19 pandemic as compared to prior to the pandemic with level of significance <0.05.

In contrast to these effects, 13 patients (35.1%) reported a negative impact of the lockdown on their dietary behavior (see Table 3). This entailed eating more unhealthy snacks while staying at home and the scarcity of gluten-free products. Five participants (13.5%) reported that they would like to have had financial support during the COVID-19 pandemic, due to the increase in expenses of the GFD. One mother stated: "Since a lot of gluten-free products were sold out, we had to buy more expensive products than we would have normally done." Patients and their parents were asked whether they wanted more or different support from their health care providers during the COVID-19 pandemic. This was not the case for all 37 patients. One parent reported that a consultation with the dietitian concerning the GFD was converted to a video-call instead of an in-person visit to the hospital, which they perceived as more convenient.

## **4. Discussion**

This cross-sectional questionnaire-based study explores the experiences of a small group of Dutch children, diagnosed with CD according to the ESPGHAN guidelines, along with their families, regarding the GFD and their disease in general during the first period of the COVID-19 pandemic. Their experiences can be utilized as learning points for the management of CD in children in general. This group of patients exhibited a generally good to high compliance to the diet before the COVID-19 pandemic. However, during the COVID-19 pandemic, an effect on eating behavior could be observed in these children. Only a very limited amount of gluten ingestion was reported. This is in contrast with an anonymous survey conducted in the United States of America, where significantly more intentional gluten intake was reported by patients with CD, as well as an impactful drop in the availability of gluten free products [17].

In contrast, in the current study, a majority of patients surprisingly felt it was easier to follow the GFD under the conditions of the lock-down, in which children consumed all meals at home. This indicates that eating outside of the home is perceived by patients as an important risk factor for gluten contamination [18]. In addition, the results revealed areas with room for improvement with regard to healthy product choices and the convenience of buying gluten-free products, which were discovered by patients due to the COVID-19 pandemic.

Overall, this study suggests that pediatric patients with CD, along with their families, appear to be moderately affected by the COVID-19 pandemic with regards to diet and patient care. The pandemic and especially its effects on the everyday life of children has been identified as a potential risk factor for unhealthy dietary patterns [10,11,15] (unpublished quantitative data from the COLC study: ClinicalTrials.gov Identifier: NCT04411511). Therefore, a main focus of this study was on the management of the GFD and general dietary habits during and prior to the COVID-19 pandemic. Interestingly, about 1/5th of patients stated that their eating patterns were healthier during the COVID-19 pandemic than before. This was attributed to having more time to cook at home. This is in contrast to findings of our research group regarding a general population of healthy children in the Netherlands (unpublished data from the COLC study). Here, a large group reported more unhealthy eating patterns during the lockdown. Among the group of children with CD, 29.7% of the patients reported eating more healthy snacks, whereas only half of this percentage reported doing so in the COLC-cohort. Besides an increase in healthy snack consumption in a subgroup of children, in the current study, 37.8% of the patients reported eating unhealthy snacks more often. This is in line with the COLC-study cohort, where approximately one in three participants reported eating

more unhealthy snacks. This emphasizes the need to promote a healthy lifestyle for all children, especially in a lockdown period.

Notably, less than half of the patients in the study reported achieving the recommended daily amount of fruit intake, prior to as well as during the pandemic. Healthy eating becomes of even greater importance in patients with a restrictive diet, such as the GFD, as it often inherently of lower nutritional quality [19]. Doctors and dieticians should therefore emphasize this during their education of patients with CD and find new strategies to encourage favorable dietary patterns in this population.

The pandemic affected grocery shopping behavior, prompting shifts such as the hoarding of products and limiting visits to supermarkets, which affected the availability of products. About half of the patients and their families took special measures to ensure that there were sufficient gluten-free products at home. Furthermore, a substantial number of patients reported that gluten-free products were indeed less available during that time, which is a potential risk factor for decreased compliance. This could possibly lead to more stress and worry in families with patients with CD or other chronic diseases that require a special diet. This should therefore receive more attention in the event of future lock-down periods.

Interestingly, as a positive outcome of the first lockdown period, some patients reported that they became more aware of a wider assortment of products due to more online grocery shopping. Due to the scarcity of products and in order to limit visits to the supermarket, they explored online resources for gluten-free products. Ideally, a health crisis should not have been necessary to make patients aware of these resources. Dieticians and other health care providers could make patients more aware of the online resources for gluten-free products. Practical information regarding how to acquire affordable, high quality gluten-free products does not seem to be readily available to all families with patients with CD, although this is an important aspect of the everyday lives of patients with CD, as well as their families.

However, the scarcity of products, as well as online grocery shopping, led to a perceived higher financial burden of the GFD during the pandemic, as reported by participants and their parents. Consequentially, parents stated that they would have liked to receive financial support during the pandemic to compensate for this. This finding should be taken into account, for example, in the form of governmental or health care insurance support, either financially or in form of resources, for patients with special dietary requirements [7]. In Jordan for example, registered patients with CD received gluten-free flour during the COVID-19 lock down [20].

With regards to patient care, it should be noted that this questionnaire was filled out by patients with a known CD diagnosis. They did not perceive disadvantages of their

care during the COVID-19 pandemic. One patient even reporting that telehealth consultations with the dietitian were more convenient than in-person visits to the hospital. The emergence of telehealth as a consequence of the pandemic should not be strictly reserved for new health crises in the future. Chronic diseases such as CD could benefit from telehealth in general clinical practice. The resources for this form of patient care have rapidly grown due to the COVID-19 crisis, but obstacles and pitfalls still need further attention. Reducing

visits to the hospital could result in reducing the disease burden of CD in the pediatric CD population. Earlier studies explored the emergence of telehealth in adult CD, with positive results, especially for young adults, and introduced best practice recommendations for introducing telehealth in pediatric gastroenterology, showing its possible benefits [13,21].

Patients that were newly diagnosed with CD during the pandemic or shortly thereafter were not included in this study. It would be worthwhile to examine how patients perceived the care they received or did not receive during the lock-down period, and how possibly delaying healthcare visits or diagnostics affected this group.

None of the 37 participating patients suffered from a proven COVID-19 infection. This cohort is too small to draw any definite conclusions regarding the prevalence of COVID-19 in patients with CD. However, these findings appear to be in line with other reports that did not find an increased risk for COVID-19 infection in patients with CD [22,23].

The current study population was very small, with an accompanying limitation that the compliance to the GFD was very high prior to the pandemic. This might indicate that not all results can be extrapolated to a larger pediatric CD population. On the other hand, the changes that were reported by these families are more likely to be related to the COVID-19 pandemic rather than other factors, as they appeared to have stable dietary habits with a good compliance to the GFD prior to the pandemic [24]. Under lockdown conditions, this possible inclusion bias may have affected how families coped with the dietary challenges of the GFD. As a result of the novelty of the situation, the questionnaire used was not validated.

However, several characteristic of the study population were fairly representative of the pediatric CD patient population in the Netherlands. It included a greater percentage of girls; the mean age was 10 years, with a range from 3 up to 18 years. The experience with the GFD and CD diagnosis had a wide range. A further strength of the study was that it only included patients with a confirmed CD diagnosis according to the ESPGHAN guidelines.

## **5. Conclusions**

In conclusion, this study creates new insight into the experiences of a small group of children with CD and their families during the first period of the current health crisis. In general, adherence to the diet appeared to be more feasible during the COVID-19 lockdown period, due to the controlled situation of eating at home. Further, patients reported that the circumstances led to more online grocery shopping which could increase the diversity of products available for patients. Although most children with CD in this cohort appeared to follow a healthier diet, attention should be given to unhealthy food habits that could have developed in patients with CD during this crisis. The natural experiment of the COVID-19 pandemic provides us with valuable information about the effects of the outbreak, as well as learning points for the management of pediatric CD in general.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/medicina59030425/s1>, Supplementary Questionnaire S1. Celiac disease and the COVID-19 pandemic in children younger than 12 years. Supplementary Table S1. Symptoms that occurred or worsened since start of the COVID-19 pandemic related either to celiac disease (CD) activity, a possible COVID-19 infection, or both (concordant symptoms).

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study

**Conflicts of Interest:** The authors declare no conflict of interest.

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**Supplementary Table S1:** Symptoms that occurred or worsened since start of the COVID-19 pandemic either related to celiac disease (CD) activity, a possible COVID-19 infection or both (concordant symptoms).

Symptom	Associated with COVID-19 or CD
Common cold	COVID-19
Coughing	COVID-19
Stuffy feeling	COVID-19
Shortness of breath or wheezing	COVID-19
Sore throat	COVID-19
Fever	COVID-19
Sneezing	COVID-19
Coughing up mucus	COVID-19
Fatigue	COVID-19 and CD (concordant symptom)
Muscle soreness	COVID-19
Chest pain	COVID-19
Diarrhea	COVID-19 and CD (concordant symptom)
Stomach ache	COVID-19 and CD (concordant symptom)
Nausea/ vomiting	COVID-19 and CD (concordant symptom)
Eye infection	COVID-19
Lost sense of smell	COVID-19
Headache	COVID-19 and CD (concordant symptom)
Constipation	CD
Bloated stomach/ flatulence	CD
Weight loss	CD
Lower energy level	CD
Red itchy rash	CD
Sores in mouth	CD



# GENERAL DISCUSSION AND SUMMARY

Celiac disease (CD) is a chronic disease with a rising prevalence worldwide affecting millions of people across the globe. CD is known to have a relatively high disease burden, in part because a strict gluten-free diet (GFD) is currently the only treatment (1). This seriously impacts daily life of patients with CD and their families, and acts as a constant reminder of their disease (2-6). Further, unintentional gluten ingestion during treatment can lead to severe symptoms, hindering participation in society for short or even longer periods of time (7-9). The effects are even more pronounced in those with early age onset, as compared to older age of onset, due to among others the usually more severe symptoms at presentation (10, 11). The overarching goal of this thesis was to support strategies aiming to reduce the disease burden of CD in children by addressing to major aims. First, by focusing on disease prevention by investigating the role of dietary factors and involved barrier disruption in CD etiology (**aim 1**). The second aim was to improve management and follow-up of CD in children by investigating challenges of the GFD beyond gluten elimination (**aim 2**).

### **Aim 1: investigating role of barrier and dietary factors in CD etiology**

The pronounced disease burden of CD and its rising prevalence make the search for preventive strategies paramount. Additionally, due to the genetic component of the disease, patients with CD often worry about passing it on to their own children. So far, most information on risk factors is based on observational studies pointing to associations. An important observational study in CD research reported an observed rapid increase in early onset CD incidence followed by a rapid decrease in incidence in children in Sweden in the 1990s likened to an epidemic (12). This “CD epidemic” was linked to changes in infant feeding practices in the same period of time in Sweden, including breast feeding duration and versus formula feeding and gluten introduction into the diet. This resulted in the hypothesis that the moment of gluten introduction into the diet during infancy, as well as the amount of gluten intake during infancy can be utilized as a modifiable factor for CD prevention. Unfortunately, large prospective studies conducted thereafter were not able to identify a strategy of early gluten introduction that altered CD risk (13, 14).

This stresses the relevance to get further insight into the possible mechanism of action of dietary factors in CD risk. A key question herein is how large amounts of gluten can come into contact with the gut-associated immune system resulting in the CD specific aberrant immune reaction. The first aim was to investigate the role of dietary factors and barrier disruption in CD etiology (see **chapters 2&3**).

Altered barrier function, may it be intrinsic or triggered by external factors, is thought to play a role in CD onset. This provides the basis for increased gliadin passage across the intestinal barrier enabling subsequent contact of gliadin with the gut-associated immune system. A high exposure of the immune system to gliadin, together with other factors such as genetic factors and/or infections may then facilitate the aberrant immune reaction described in CD to occur (15, 16).

In order to investigate whether intestinal damage is not only a hallmark of CD manifestation, but also present prior to CD onset, we investigated epithelial damage by measuring a biomarker for small intestinal damage, *i.e.* intestinal fatty acid binding protein (I-FABP) in a unique cohort including children with a high risk to develop CD. Further, the effect of dietary factors on epithelial permeability was investigated using a well-established *in vitro* cell culture model (see **chapter 3**).

In **chapter 2**, I-FABP was measured in children with a high risk to develop CD, in blood samples taken early in life and before onset of CD. In this unique European cohort, children were followed for at least 10 years and follow-up is still ongoing to monitor who eventually develops CD. Differences in I-FABP concentrations were found in the first year of life when children that developed early onset CD were compared to those that did not develop CD within ten years of life. This supports the hypothesis that intestinal damage is present prior to disease onset and may be a risk factor. This different evolution of enterocyte damage in the first year of life of children that develop CD early in life could be influenced by external factors. Thus, the association between intestinal damage and potential modifying factors should be further investigated including infections, vaccinations, and feeding practices such as breastfeeding including its cessation, introduction of solid food and gluten and dietary patterns in general, the latter being a focus point of this thesis.

As CD onset can occur very early in life, preventive strategies must be applied in infancy. It must be taken into consideration, that a large number of children can be considered at-risk to develop CD, *i.e.* being carriers of an HLA-DQ 2 or 8 allele (17-19). Yet only a small fraction of them eventually develop CD. In order to be efficient, preventive measures must be applied to a large group of children that would naturally never develop CD and preferably should pose a low burden to the child and their family and ideally yield a general health benefit. Beneficial dietary advices therefore represent a good candidate target to explore in this context.

Components of the Western diet are thought to be a potential risk factor for CD development and are hypothesized to play a role in the increasing prevalence of CD. In **chapter 3**, we investigated the effect of dietary sugars alone and in combination with digested gliadin, the immunogenic component of gluten and dietary sugars on enterocytes and barrier function in a well-established *in vitro* Caco-2 model.

Exposure of the epithelial cell monolayer to gliadin, glucose, and fructose separately and in combination for one hour (*i.e.* mimicking small intestinal exposure), did lead to an increase in intestinal permeability. Glucose and fructose were used as key components of the Western diet, thought to be risk factors for CD development. In line with other studies, gliadin significantly reduced the trans-epithelial electrical resistance (TEER) pointing to increased intestinal permeability. Exposure of the Caco-2 cells to both sugars also led to an increase in permeability and separate exposure to glucose even lead to an increased flux of a large molecule (FITC-D4) across the membrane. Interestingly, the combination of digested gliadin with monosaccharides attenuated the negative effects on barrier function to some extent. This is important to take into account for future research indicating that translation of the model to the real life physiological condition asks to not only to look at dietary components separately, but also in realistic combinations resembling true dietary intake.

Overall, the findings support the notion that dietary factors can compromise intestinal barrier function and integrity. This underscores the importance of dietary components in intestinal barrier dysfunction and their potential implications for CD and related disorders. Concerning CD risk, gliadin and monosaccharides are interesting candidates to examine, but future research should be expanded to additional components of the “Western diet” separately and in combination with each other. It likely is too simplistic to consider the separate effect on barrier integrity of any component, as their combination can in fact lead to altered effects as shown in this study. Moreover, processing of the different components in a meal or the combination with food additives could also alter the individual separate effects of dietary components. Future research should therefore focus more on simulating the effects of a true meal (food intake) rather than considering the effects of separate components only. Understanding the underlying mechanisms and potential interactions between gliadin and monosaccharides is crucial. Further research should investigate differences in gene expression and cellular pathways to gain more insights into these interactions. Additionally, studying gut maturation, functionality, and damage in early life may provide a better understanding of CD development and other risk factors.

To this end, a basic model such as the Caco-2 model can be used to further understand the underlying cellular pathways involved in the interplay between barrier function and dietary components. Next, it would be interesting to add the genetic component of CD and compare the reaction of the intestinal barrier to dietary components between people with and without genetic susceptibility to develop CD. Use of host-specific organoids could be an interesting opportunity to check if responses may differ in people with a genetic susceptibility to develop CD (20-23). Further, when exploring the role of the barrier in patients or at-risk

cohorts, investigation of epithelial damage and paracellular permeability should be analyzed together. An interesting option could be for instance combining the use of the biomarker I-FABP with a functional test investigating permeability such as a sugar absorption test (24). This would also tackle the aforementioned challenge of investigating separate components in a laboratory setting versus real food intake in a study with human subjects. This would allow to move from analyzing separate dietary components to investigating the diet and dietary patterns in its entirety. Furthermore, it should be noted that also the intestinal mucus layer and the commensal microbiota among other factors contribute to a well-functioning intestinal barrier.

As highlighted in this thesis, the findings on barrier function and damage should be combined with information on dietary intake. The steps that have to be taken into account are nevertheless numerous and will be time-consuming, but diet as a modality in CD risk remains in my opinion a pivotal target to focus on future preventive strategies. In this regard, it should not be dismissed that optimizing diet and dietary education could also have an overall health benefit for families and future generations.

Identifying such potential targets for CD prevention can occur via different routes. Understanding the etiology of the disease and the mechanism of action of the different triggers for disease onset can disclose potential targets. Alongside this, observational studies yielding risk factors that may play a role can deliver potential future targets as well. Indeed, dietary factors have emerged as potential targets for prevention mainly through observational studies that identified certain dietary patterns as potential risk or protecting factors for CD (12, 25).

## **Aim 2: Disease management in pediatric CD**

Identifying and testing potential preventive strategies in practice is a long process, which does not directly yield benefits for the current CD population. Therefore, improving treatment for current patients is very important. The research efforts presented in this thesis therefore span from investigating modifiable risk factors for CD to improvement of CD management, focusing on the role of dietary factors. In this regard, the second aim of this thesis was to examine challenges of the GFD beyond the elimination of gluten (see **chapters 4 to 6**).

Whereas the European guidelines on diagnosis of pediatric CD are quite extensive and have been updated twice in the past decade, guidelines on management and follow-up of CD were nonexistent until very recently when the Special Interest Group in CD of ESPGHAN published a position paper on CD management for the

first time in 2022 (26). An important takeaway from this paper was a substantial knowledge gap in evidence-based strategies for the follow-up of pediatric CD. Specifically, the optimal timing and frequency of serological testing remain uncertain. Factors that are assessable through serological testing next to CD auto-antibodies, including micronutrient values, blood count, thyroid hormone levels and liver function tests lack adequate evidence on frequency of aberrations. Furthermore, understanding of the clinical implications of short- and long-term aberrations of these parameters in children with CD is limited.

The lack of evidence highlighted in the recent publication of ESPGHAN is in line with the results of the literature review included in this thesis (**chapter 4**). There, the existing evidence for the occurrence of nutrient deficiencies during CD follow up in adults and pediatric patients was examined. In the review, we aimed to create an overview on dietary intake of several vitamins and micronutrients in patients with CD while following a GFD. This is important to examine, as the risk to develop nutrient deficiencies over time while following a GFD are not well investigated irrespective of compliance to the GFD.

The results of the literature review as well as of the retrospective pediatric CD cohort study conducted in the MUMC+ both provided evidence that newly diagnosed patients as well as those following a GFD can frequently exhibit nutrient deficiencies especially concerning vitamin D, iron and ferritin deficiency (**chapters 4 & 5**). These can occur even after following a GFD for a long time (**chapter 5**). In order to assess the importance of these identified nutrient deficiencies, the possible clinical consequences in this specific patient group must be further examined. Importantly, a less frequently encountered deficiency such as folate deficiency may nevertheless exert a substantial influence on growth and development, thus necessitating ongoing monitoring despite its low prevalence. Moreover, the duration of nutrient recovery may have significant clinical implications. Research should explore whether accelerated restoration of nutrient status is beneficial for short and long- term clinical course. For instance through incorporating nutrient supplementation alongside with dietary guidance at the moment of CD diagnosis. The nutrient deficiencies often found at the time of CD diagnosis can be considered an initial impact on short and long-term growth and development. This is highlighted by the increased risk of patients with CD to develop osteoporosis or exhibit bone fractures later in life. Maintenance of optimal levels of calcium and vitamin D must therefore be prioritized in patients with CD following diagnosis. Detecting and correcting mild deficiencies or values near the cut-off points, even without noticeable symptoms, may be crucial during CD patient follow-up, emphasizing the distinct importance of nutritional status in this population.

Gaining clarity on these aspects could lead to a revised understanding of the importance of nutritional status in this specific patient group as compared to the general population. Future multinational cohort studies should be conducted to account for variations in dietary habits and types of medical care across different patient groups, ranging from care by general practitioners to specialized tertiary centers. Additionally, researchers should pay closer attention to methodological considerations when assessing nutrient deficiencies including the selection of measurement techniques and appropriate reference values, in order to accurately differentiate between patients with sub-optimal nutrient levels and those with severe deficiencies. This information can then be used to reach a consensus on the necessity and frequency of follow-up of nutritional status in children with CD following a GFD as part of their clinical management. As frequency between different deficiencies varies greatly, it could be considered to categorize nutrients in order of prevalence of their deficiency and plan monitoring accordingly.

The presented results from **chapters 4** and **5** showed that the GFD can be a risk factor to develop nutrient deficiencies through inadequate dietary intake. This also underscores the importance of good dietary counseling and education of patients with CD and their families by not only focusing on proper elimination of gluten from the diet, but ensuring at the same time adequate dietary intake of other nutrients as well. In the literature review, we also showed how challenging sufficient dietary intake remains while following a GFD. Moreover, enhancing the quality of gluten-free products could provide additional benefits. Possibilities for fortification and stricter rules and regulations on nutritional composition of gluten-free products could be a part of health policies supporting patients with CD and others following a GFD alike. In some countries, up to 7% of individuals report following a GFD, surpassing the overall diagnosed prevalence of CD estimated to be at 1-2% (27-29). Consequently, this trend has led to an increased demand for gluten-free products, prompting a rapid expansion in the availability and quality of such products. Next to this being an attractive opportunity for the food industry, it also makes the importance of regulation of quality of these products even more important (30). Falcomer *et al.* studied public policies supporting patients with CD worldwide. They concluded that overall policies regarding gluten-free meals, health service support, and financial incentives for patients with CD were poor, albeit strongest in Europe in global comparison (31). Patient education regarding diet quality and potential deficiencies is crucial. Simultaneously, it is essential to acknowledge the shared responsibilities of healthcare providers, the government, and the food industry in supporting patients' success. Therefore, it is crucial to engage all stakeholders in fulfilling their responsibilities to maintain the nutritional quality of the gluten-free diet.

Patient education and counseling should extend beyond the moment of diagnosis, where the elimination of gluten is of utmost importance, and remain a crucial aspect throughout the disease follow-up. This is particularly significant for children, as their dietary needs and challenges can evolve from infancy to young adulthood. The questionnaire study completed during the COVID-19 pandemic also showed other aspects of the GFD that need further attention (chapter 6). Eating outdoors was found to be the main source for unintentional gluten ingestion, which can lead to increased anxiety and changing of social behavior. Thus, patients should be equipped with resources and tools that empower them to confidently maintain a gluten-free lifestyle in their everyday routines. This may involve practical elements such as developing effective communication skills to ascertain whether a restaurant fully comprehends the precise implications of “gluten-free” for individuals with CD. It also includes providing educational materials to schools and teachers, emphasizing the significance of the GFD and the prevention of cross-contamination in the school or daycare setting and during field trips. As a positive byproduct of the COVID-19 pandemic, patients and their parents reported the advantages of online shopping for gluten-free products (chapter 6). Such online possibilities should be included in future patient education. Providing resources and tools to patients with CD on how to manage their diet should be an ongoing process aiming to improve quality of life and lowering disease burden. The challenges of living with CD and following a GFD are still present in daily life of patients with CD, their families and their health care providers. Besides preventing CD, we can benefit from focusing on overall health and health related quality of life of patients with CD. The findings in this thesis show that focusing on nutrition in a broader sense than merely omitting gluten from the diet is important and may improve the overall strategy to reduce the risk of CD onset and improving disease course and quality of life after diagnosis.

### **Diet as the common denominator – harnessing diet in CD prevention and management**

Diet plays an important role in health and disease (32-34). The potential of dietary factors as risk factors for disease onset or progression is especially known in non-communicable diseases, such as fat and sugar intake in association with type 2 diabetes and metabolic dysfunction-associated steatotic liver disease (MASLD), but also with chronic inflammatory disorders such as Inflammatory Bowel Disease (IBD) (34-37). Thereby, diet can also be used either as a preventive or therapeutic strategy. A par excellence prime example for this represents the GFD for the treatment of CD.

It is widely recognized that dietary factors, particularly gluten exposure, have a significant contribution in CD pathogenesis. Moreover, it is hypothesized that

a Western diet may contribute to the onset of CD (see **chapter 3**). This growing recognition of utilizing specific dietary guidelines as part of disease management is evident in various other conditions such as the adoption of low sodium diets for patients with chronic kidney disease and/or hypertension (38). Additionally, elimination diets, such as the GFD, are commonly implemented for individuals with food intolerances or allergies, even extending to those without a formal CD diagnosis due to perceived benefits associated with its adoption (9).

Moving forward, we should further investigate how to harness the power of dietary factors in favor of disease prevention and improved disease management.

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# ADDENDUM



SUMMARY  
SAMENVATTING  
ZUSAMMENFASSUNG

## Summary

Celiac disease (CD) is a chronic disease with a rising prevalence worldwide affecting millions of people across the globe. CD is known to have a relatively high disease burden, in part because a strict gluten-free diet (GFD) is currently the only treatment. This seriously impacts daily life of patients with CD and their families, and acts as a constant reminder of their disease. Further, unintentional gluten ingestion during treatment can lead to severe symptoms, hindering participation in society for short or even longer periods of time. The effects can be even more pronounced in those with early age onset, due to among others the usually more severe symptoms at presentation. The overarching goal of this thesis was to explore strategies aiming to reduce the disease burden of CD in children by addressing two major aims. First, by focusing on disease prevention by investigating the role of dietary factors and involved barrier disruption in CD etiology (**aim 1**). The second aim was to improve management and follow-up of CD in children by investigating challenges of the GFD beyond gluten elimination (**aim 2**).

### Aim 1: investigating role of barrier and dietary factors in CD etiology

Observational studies have pointed to associations between several potential risk factors and CD. The timing and amount of gluten introduction during infancy were hypothesized to be modifiable factors for CD prevention, but subsequent large prospective studies did not find a strategy for reducing CD risk. Thus further insight into the possible mechanism of action of dietary factors in CD risk is needed. A key question herein is how large amounts of gluten can come into contact with the gut-associated immune system resulting in the CD specific aberrant immune reaction. The first aim was to investigate the role of dietary factors and barrier disruption in CD etiology (see **chapters 2 & 3**).

Altered barrier function, may it be intrinsic or triggered by external factors, is thought to play a role in CD onset. This provides the basis for increased gliadin passage across the intestinal barrier enabling subsequent contact of gliadin with the gut-associated immune system. A high exposure of the immune system to gliadin, together with other factors such as genetic factors and/or infections may then facilitate the aberrant immune reaction described in CD to occur.

In order to investigate whether intestinal damage is not only a hallmark of CD manifestation, but also present prior to CD onset, we investigated epithelial damage by measuring a biomarker for small intestinal damage, *i.e.* intestinal fatty acid binding protein (I-FABP) in a unique cohort including children with a high risk

to develop CD. Further, the effect of dietary factors on epithelial permeability was investigated using a well-established *in vitro* cell culture model (see **chapter 3**).

In **chapter 2**, I-FABP was measured in children with a high risk to develop CD, in blood samples taken early in life and before onset of CD. In this unique European cohort, children were followed for at least 10 years and follow-up is still ongoing to monitor who eventually develops CD. Differences in I-FABP concentrations were found in the first year of life when children that developed early onset CD were compared to those that did not develop CD within ten years of life. This supports the hypothesis that intestinal damage is present prior to disease onset and may be a risk factor.

In **chapter 3**, we investigated the effect of dietary sugars alone and in combination with digested gliadin, the immunogenic component of gluten and dietary sugars on enterocytes and barrier function in a well-established *in vitro* Caco-2 model. Here, exposure to digested gliadin, glucose, and fructose led to increased intestinal permeability. Interestingly, the combination of gliadin with monosaccharides attenuated the negative effects on barrier function to some extent. Overall, these findings support the notion that dietary factors can compromise intestinal barrier function and integrity. This underscores the importance of dietary components in intestinal barrier dysfunction and their potential implications for CD and related disorders.

## **Aim 2: Disease management in pediatric CD**

Identifying and testing potential preventive strategies in practice is a long process, which does not directly yield benefits for the current CD population. Therefore, improving treatment for current patients is very important. The research efforts presented in this thesis therefore span from investigating modifiable risk factors for CD to improvement of CD management, focusing on the role of dietary factors. In this regard, the second aim of this thesis was to examine challenges of the GFD beyond the elimination of gluten (**chapter 4 to 6**).

There is a substantial knowledge gap in evidence-based strategies for the follow-up of pediatric CD. Specifically, the optimal timing and frequency of serological testing remain uncertain. Factors that are assessable through serological testing next to CD auto-antibodies, including micronutrient values, blood count, thyroid hormone levels and liver function tests lack adequate evidence on frequency of aberrations. Furthermore, understanding of the clinical implications of short- and long-term aberrations of these parameters in children with CD is limited.

The literature review included in this thesis (**chapter 4**) examined the existing evidence for the occurrence of nutrient deficiencies during CD follow-up in adults and pediatric patients. Additionally, we created an overview on dietary intake of several vitamins and micronutrients in patients with CD while following a GFD. This review as well as of the retrospective pediatric CD cohort study conducted in the Maastricht UMC+ both provided evidence that newly diagnosed patients as well as those following a GFD can frequently exhibit nutrient deficiencies especially concerning vitamin D, iron and ferritin deficiency (**chapters 4 & 5**). These can occur even after following a GFD for a long time (**chapter 5**). Moreover, the presented results from **chapters 4** and **5** showed that the GFD can be a risk factor to develop nutrient deficiencies through inadequate dietary intake. This also underscores the importance of good dietary counseling and education of patients with CD and their families by not only focusing on proper elimination of gluten from the diet, but ensuring at the same time adequate dietary intake of other nutrients as well. In the literature review, we also showed how challenging sufficient dietary intake remains while following a GFD.

In line with this, the questionnaire study completed during the COVID-19 pandemic also showed other aspects of the GFD that need further attention (**chapter 6**). Eating outside of the home was found to be the main source for unintentional gluten ingestion, which can lead to increased anxiety and changing of social behavior. As a positive byproduct of the COVID-19 pandemic, patients and their parents reported the advantages of online shopping for gluten-free products (**chapter 6**). The challenges of living with CD and following a GFD are still present in daily life of patients with CD, their families and their health care providers.

## **Diet as the common denominator – harnessing diet in CD prevention and management**

This thesis shows that focusing on nutrition in a broader sense than merely omitting gluten is important and may improve the overall strategy to reduce the risk of CD onset and improving disease course and quality of life after diagnosis.

In order to assess the importance of the identified nutrient deficiencies associated with CD, the possible clinical consequences in this specific patient group must be further examined. Gaining clarity on these aspects could lead to a revised understanding of the importance of nutritional status in this specific patient group as compared to the general population. This information can then be used to reach a consensus on the necessity and frequency of follow-up of nutritional status in children with CD following a GFD as part of their clinical management. As frequency between different deficiencies varies greatly, it could be considered

to categorize nutrients in order of prevalence of their deficiency and plan monitoring accordingly. Simultaneously, it is essential to acknowledge the shared responsibilities of healthcare providers, the government, and the food industry in supporting patients' success. Therefore, it is crucial to engage all stakeholders in fulfilling their responsibilities to maintain the nutritional quality of the GFD.

Diet plays an important role in health and disease. The potential of dietary factors as risk factors for disease onset or progression is especially known in non-communicable diseases, such as fat and sugar intake in association with type 2 diabetes and metabolic dysfunction-associated steatotic liver disease (MASLD), but also with chronic inflammatory disorders such as Inflammatory Bowel Disease. Thereby, diet can also be used either as a preventive or therapeutic strategy. A par excellence prime example for this represents a GFD for the treatment of CD.

It is widely recognized that dietary factors, particularly gluten exposure, have a significant contribution in CD pathogenesis. Moreover, it is hypothesized that a Western diet may contribute to the onset of CD (**chapter 3**). Therefore, dietary advice represents a promising target for CD prevention, as components of the Western diet are hypothesized to be risk factors for CD development. In addition, future research should be expanded to additional components of the Western diet separately and in combination with each other. It likely is too simplistic to consider the separate effect on barrier integrity of any component, as their combination can in fact lead to altered effects as shown in this thesis.

Moving forward, we should further investigate how to harness the power of dietary factors in favor of disease prevention and improved disease management.

## Samenvatting

Coeliakie (CD), ofwel gluten intolerantie, is een chronische ziekte die miljoenen mensen over de hele wereld treft. CD staat bekend om zijn relatief hoge ziekteleast, deels omdat de enige behandeling op dit moment een strikt glutenvrij dieet (GVD) is. Dit heeft een ernstige impact op het dagelijkse leven van patiënten met CD en hun families mede omdat het een voortdurende herinnering aan de ziekte is.

Het overkoepelende doel van dit proefschrift was om strategieën te ondersteunen die gericht zijn op het verminderen van de ziekteleast van CD bij kinderen. Ten eerste, door te focussen op ziektepreventie door de rol van dieetfactoren en betrokken barrièrerestoring in de etiologie van CD te onderzoeken (**doel 1**). Het tweede doel was om het management en de follow-up van CD bij kinderen te verbeteren door de uitdagingen van de GVD naast gluteneliminatie te onderzoeken (**doel 2**).

### **Doel 1: onderzoek naar de rol van barrière- en dieetfactoren in het ontstaan van CD**

De uitgesproken ziekteleast van CD en de stijgende prevalentie maken de zoektocht naar preventieve strategieën van het allergrootste belang. Bovendien maken patiënten met CD zich, vanwege de genetische component van de ziekte, vaak zorgen over het doorgeven van de ziekte aan hun eigen kinderen.

Observationele studies hebben verschillende mogelijke risicofactoren ontdekt voor het ontwikkelen van CD. Er werd gesteld dat het moment van introductie van gluten in het dieet van de baby, en ook de hoeveelheid van gluten die wordt gegeven aangepast kan worden om het ontstaan van CD mogelijk te voorkomen. Echter hebben latere grote prospectieve studies geen strategie gevonden om het risico op CD te verminderen. Welke rol dieetfactoren in het risico op CD spelen en het achterliggende werkingsmechanisme, moet dus nog verder worden onderzocht. Een belangrijke vraag hierin is hoe grote hoeveelheden gluten in contact kunnen komen met het afweersysteem in de darm, waar vervolgens de afwijkende afweerreactie kan plaatsvinden die kenmerkend is voor CD.

Het eerste doel van dit proefschrift was om de rol van dieetfactoren en barrièrerestoring in het ontstaan van CD te onderzoeken (zie **hoofdstukken 2&3**).

Men denkt dat een verstoorde barrierefunctie, of deze nu intrinsiek is of wordt uitgelokt door externe factoren, een rol speelt bij het ontstaan van CD. Dit vormt de basis voor een verhoogde passage van gliadine door de darmbarrière, waardoor gliadine in contact kan komen met het darm-geassocieerde immuunsysteem. Een

hoge blootstelling van het immuunsysteem aan gliadine, samen met andere factoren zoals genetische factoren en/of infecties, kan dan de afwijkende immuunreactie zoals beschreven bij CD bevorderen.

Om te onderzoeken of darmbeschadiging niet alleen een kenmerk is van de manifestatie van CD, maar ook aanwezig is vóór het begin van CD, onderzochten we epitheliale schade door het meten van een kenmerk voor schade aan de dunne darm. Dit betrof het meten van 'intestinale vetzuurbindingende proteïne' (I-FABP) in een uniek cohort met kinderen die een hoog risico hadden om CD te ontwikkelen. Verder werd het effect van voedingsfactoren op de epitheliale permeabiliteit onderzocht met behulp van een bekend *in vitro* celkweekmodel (zie **hoofdstuk 3**).

In **hoofdstuk 2** werd I-FABP gemeten bij kinderen met een hoog risico op het ontwikkelen van CD, in bloedmonsters die op jonge leeftijd en vlak voor het begin van CD werden afgenoem. In dit unieke Europese cohort werden de kinderen minstens 10 jaar gevolgd en de follow-up is nog steeds gaande om te controleren wie uiteindelijk CD ontwikkelt. Er werden verschillen in I-FABP-concentraties gevonden in het eerste levensjaar wanneer kinderen die op jonge leeftijd CD ontwikkelden werden vergeleken met kinderen die geen CD ontwikkelden binnen tien jaar na hun geboorte. Dit ondersteunt de hypothese dat darmbeschadiging al aanwezig is voordat de ziekte begint en een risicofactor kan zijn.

In **hoofdstuk 3** onderzochten we het effect van suikers met en zonder combinatie van verterd gliadine - het immunogene bestanddeel van gluten - op enterocyten en de barrierefunctie in een celkweek-model. Hier leidde blootstelling aan verterd gliadine, glucose en fructose tot een verhoogde darmpermeabiliteit. Interessant is dat de combinatie van gliadine met monosachariden de negatieve effecten op de barrierefunctie tot op zekere hoogte verminderde. Deze bevindingen ondersteunen in het algeheel het idee dat voedingsfactoren de barrierefunctie en -integriteit van de darm in gevaar kunnen brengen. Dit onderstreept het belang van voedingscomponenten in de disfunctie van de darmbarrièrē en hun mogelijke implicaties voor CD en aanverwante aandoeningen.

## **Doeel 2: Behandeling en begeleiding van kinderen met CD**

Het identificeren en testen van mogelijke preventieve strategieën in de praktijk is een lang proces, dat niet direct voordeelen oplevert voor de huidige patiënten populatie met CD. Daarom is het verbeteren van de behandeling voor de huidige patiënten erg belangrijk. De onderzoeksinspanningen in dit proefschrift strekken zich daarom uit van het onderzoeken van aanpasbare risicofactoren voor CD tot het verbeteren van het CD-management, waarbij de nadruk ligt op de rol

van dieetfactoren. In dit opzicht was het tweede doel van dit proefschrift om uitdagingen van het GVD te onderzoeken die verder gaan dan de eliminatie van gluten (**hoofdstuk 4 tot 6**).

Er is een substantieel gat qua kennis in wetenschappelijk onderbouwde strategieën voor de follow-up van pediatrische CD. Met name de optimale timing en frequentie van serologische testen blijven onzeker. Factoren die beoordeeld kunnen worden door middel van serologische testen naast auto-antilichamen tegen CD, waaronder waarden van micronutriënten, bloedbeeld, schildklierhormoonspiegels en leverfunctietesten, hebben onvoldoende bewijs voor de frequentie van afwijkingen. Bovendien is het begrip van de klinische betekenis van korte en lange termijn-afwijkingen van deze parameters bij kinderen met CD beperkt.

De literatuurstudie in dit proefschrift (**hoofdstuk 4**) onderzocht het bestaande bewijs voor het voorkomen van nutriëntendeficiënties tijdens de follow-up van CD bij volwassenen en pediatrische patiënten. Daarnaast hebben we een overzicht gemaakt van de inname van verschillende vitamines en micronutriënten via het dieet bij patiënten met CD die een GVD volgen. Zowel dit overzicht als de retrospectieve CD-kindercohortstudie in het Maastricht UMC+ leverden bewijs dat nieuw gediagnosticeerde patiënten en patiënten die een GVD volgen vaak tekorten aan voedingsstoffen vertonen, vooral met betrekking tot vitamine D-, ijzer- en ferritin tekorten (**hoofdstukken 4 en 5**). Deze kunnen zelfs optreden na het langdurig volgen van een GVD (**hoofdstuk 5**). Bovendien toonden de gepresenteerde resultaten van **hoofdstuk 4 en 5** aan dat het GVD een risicofactor kan zijn voor het ontwikkelen van nutriëntendeficiënties door inadequaat voedselinname. Dit onderstreept ook het belang van goede dieetadviesering en -educatie van patiënten met CD en hun families, door niet alleen te focussen op de juiste eliminatie van gluten uit het dieet, maar tegelijkertijd ook te zorgen voor een adequate inname van andere voedingsstoffen. In de literatuurstudie toonden we ook aan hoe uitdagend voldoende inname van voedingsstoffen blijft tijdens het volgen van een GVD.

In lijn hiermee liet het vragenlijsonderzoek dat werd uitgevoerd tijdens de COVID-19 pandemie ook andere aspecten van het GVD zien die verdere aandacht nodig hebben (**hoofdstuk 6**). Buiten de deur eten bleek de belangrijkste bron te zijn voor onbedoelde gluteninname, wat kan leiden tot verhoogde angst en verandering van sociaal gedrag. Als positief bijproduct van de COVID-19 pandemie meldden patiënten en hun ouders de voordelen van online winkelen voor glutenvrije producten (**hoofdstuk 6**). De uitdagingen van het leven met CD en het volgen van een GVD zijn nog steeds aanwezig in het dagelijkse leven van patiënten met CD, hun families en hun zorgverleners.

## **Conclusie: voeding inzetten bij preventie en behandeling van CD**

Dit proefschrift toont aan dat focussen op voeding in een bredere zin dan alleen het weglaten van gluten belangrijk is en de algemene strategie kan verbeteren om het risico op het ontstaan van CD te verminderen en het ziektebeloop en de levenskwaliteit na de diagnose te verbeteren.

Om het belang van de nutriëntendeficiënties geassocieerd met CD te beoordelen, moeten de mogelijke klinische gevolgen in deze specifieke patiëntengroep verder worden onderzocht. Het verkrijgen van duidelijkheid over deze aspecten zou kunnen leiden tot een herzien begrip van het belang van de voedingsstatus in deze specifieke patiëntengroep in vergelijking met de algemene bevolking. Deze informatie kan dan gebruikt worden om een consensus te bereiken over de noodzaak en frequentie van opvolging van de voedingsstatus bij kinderen met CD die een GVD volgen als onderdeel van hun klinisch management. Aangezien de frequentie van verschillende deficiënties sterk varieert, kan overwogen worden om voedingsstoffen te categoriseren in volgorde van prevalentie van hun deficiëntie en de monitoring dienovereenkomstig te plannen. Tegelijkertijd is het essentieel om de gedeelde verantwoordelijkheid van zorgverleners, de overheid en de voedingsindustrie te erkennen bij het ondersteunen van het succes van patiënten. Daarom is het cruciaal om alle belanghebbenden te betrekken bij het vervullen van hun verantwoordelijkheden om de voedingskwaliteit van het GVD te handhaven.

Voeding speelt een belangrijke rol bij gezondheid en ziekte. Het potentieel van voedingsfactoren als risicofactoren voor het ontstaan of de progressie van ziekten is vooral bekend bij niet-overdraagbare ziekten, zoals vet- en suikerinname in verband met type 2-diabetes en leverziekte, maar ook bij chronische ontstekingsziekten zoals inflammatoire darmziekten (IBD). Daarbij kan voeding ook worden gebruikt als preventieve of behandelende strategie. Een uitstekend voorbeeld hiervan is het GVD voor de behandeling van CD.

Het wordt algemeen erkend dat dieetfactoren, in het bijzonder de blootstelling aan gluten, een belangrijke bijdrage leveren aan de pathogenese van CD. Bovendien wordt verondersteld dat een westerse dieet kan bijdragen aan het ontstaan van CD (hoofdstuk 3). Daarom is voedingsadvies een veelbelovend doelwit voor de preventie van CD, omdat verondersteld wordt dat onderdelen van het westerse dieet risicofactoren zijn voor de ontwikkeling van CD. Daarnaast moet toekomstig onderzoek worden uitgebreid naar aanvullende componenten van het “westerse dieet” afzonderlijk en in combinatie met elkaar. Het is waarschijnlijk te simplistisch om te kijken naar het afzonderlijke effect op de barrière-integriteit van een component, omdat hun combinatie in feite kan leiden tot veranderde effecten, zoals aangetoond in dit proefschrift.

In de toekomst moeten we verder onderzoeken hoe we de kracht van voedingsfactoren kunnen inzetten ten gunste van ziektepreventie en een beter ziektebeheer.

## **Zusammenfassung**

Zöliakie (CD) oder Gluten Unverträglichkeit ist eine chronische Krankheit, von der Millionen Menschen auf der ganzen Welt betroffen sind. Zöliakie ist für ihre relativ hohe Krankheitslast bekannt, was zum Teil darauf zurückzuführen ist, dass die einzige derzeit verfügbare Behandlungsmethode eine strikte Gluten Freie Diät (GFD) ist. Dies hat schwerwiegende Auswirkungen auf das tägliche Leben der CD-Patienten und ihrer Familien, auch weil sie ständig an die Krankheit erinnert werden.

Das übergeordnete Ziel dieser Arbeit bestand darin, Strategien zu unterstützen, die darauf abzielen, die Krankheitslast von CD bei Kindern zu verringern. Erstens sollte die Krankheitsprävention im Vordergrund stehen, indem die Rolle von Ernährungsfaktoren und Barrierestörungen in der Ätiologie der CD untersucht wurde (**Ziel 1**). Das zweite Ziel bestand darin, die Behandlung und Nachsorge von CD bei Kindern zu verbessern, indem die Herausforderungen der GFD zusätzlich zur Gluteneliminierung untersucht wurden (**Ziel 2**).

### **Ziel 1: Untersuchung der Rolle von Barriere- und Ernährungsfaktoren bei der Entwicklung von CD**

Die ausgeprägte Krankheitslast der CD und die steigende Prävalenz machen die Suche nach Präventionsstrategien von größter Bedeutung. Aufgrund der genetischen Komponente der Krankheit machen sich CD-Patienten außerdem häufig Sorgen, dass sie die Krankheit an ihre eigenen Kinder weitergeben könnten.

In Beobachtungsstudien wurden mehrere mögliche Risikofaktoren für die Entwicklung von CD entdeckt. Es wurde festgestellt, dass der Zeitpunkt der Einführung von Gluten in die Ernährung des Babys sowie die Menge an Gluten, die dem Kind verabreicht wird, eine Rolle spielen. Es wurde vermutet, dass der Zeitpunkt der Einführung von Gluten in die Ernährung des Babys und auch die Menge des verabreichten Glutens angepasst werden könnten, um die Entwicklung von CD möglicherweise zu verhindern. In nachfolgenden großen prospektiven Studien wurde jedoch keine Strategie zur Verringerung des CD-Risikos gefunden. Welche Rolle Ernährungsfaktoren für das CD-Risiko spielen und welcher Wirkmechanismus ihnen zugrunde liegt, muss also weiter untersucht werden. Eine Schlüsselfrage ist dabei, wie große Mengen Gluten mit dem Immunsystem im Darm in Kontakt kommen können, wo dann die für CD charakteristische abnorme Immunreaktion stattfinden kann.

Das erste Ziel dieser Arbeit war es, die Rolle von Ernährungsfaktoren und Barrierestörungen bei der Entstehung von CD zu untersuchen (**siehe Kapitel 2 und 3**). Es wird angenommen, dass eine gestörte Barrierefunktion, sei es intrinsisch oder durch externe Faktoren ausgelöst, eine Rolle bei der Entwicklung von CD spielt. Dies bildet die Grundlage für eine erhöhte Passage von Gliadin durch die Darmbarriere, wodurch Gliadin mit dem darmassoziierten Immunsystem interagieren kann.

Eine hohe Aussetzung des Immunsystems gegenüber Gliadin kann dann zusammen mit anderen Faktoren wie genetischen Faktoren und/oder Infektionen die bei CD beschriebene abnorme Immunreaktion fördern.

Um herauszufinden, ob die Schädigung des Darms nicht nur ein Kennzeichen für die Manifestation der CD ist, sondern auch schon vor dem Ausbruch der CD vorhanden ist, untersuchten wir die Epithelschädigung durch Messung eines Kennzeichens für die Schädigung des Dünndarms. Dazu wurde das „intestinale Fettsäurebindungsprotein“ (I-FABP) in einer einzigartigen Kohorte von Kindern mit einem hohen Risiko für die Entwicklung von CD gemessen. Außerdem wurde die Auswirkung von Ernährungsfaktoren auf die Epithelpermeabilität anhand eines bekannten In-vitro-Zellkulturmodells untersucht (**siehe Kapitel 3**).

In Kapitel 2 wurde I-FABP bei Kindern mit hohem CD-Risiko in Blutproben gemessen, die in einem frühen Alter und kurz vor dem Ausbruch der CD entnommen wurden. In dieser einzigartigen europäischen Kohorte wurden die Kinder mindestens 10 Jahre lang beobachtet, und die Nachbeobachtung wird fortgesetzt, um zu verfolgen, wer schließlich CD entwickelt. Im ersten Lebensjahr wurden Unterschiede in der I-FABP-Konzentration festgestellt, wenn Kinder, die in jungen Jahren eine CD entwickelten, mit denen verglichen wurden, die innerhalb von 10 Jahren nach der Geburt keine CD entwickelten. Dies unterstützt die Hypothese, dass Darmschäden bereits vor Ausbruch der Krankheit vorhanden sind und einen Risikofaktor darstellen können.

In Kapitel 3 untersuchten wir die Wirkung von Zuckern mit und ohne Kombination von verdautem Gliadin - der immunogenen Komponente von Gluten - auf Enterozyten und die Barrierefunktion in einem Zellkulturmodell. Hier führte die Exposition gegenüber verdautem Gliadin, Glukose und Fruktose zu einer erhöhten intestinalen Permeabilität. Interessanterweise reduzierte die Kombination von Gliadin mit Monosacchariden die negativen Auswirkungen auf die Barrierefunktion bis zu einem gewissen Grad. Insgesamt unterstützen diese Ergebnisse die Idee, dass Ernährungsfaktoren die Barrierefunktion und Integrität des Darms beeinträchtigen können. Dies unterstreicht die Bedeutung von Nahrungsbestandteilen für die

Dysfunktion der Darmbarriere und ihre potenziellen Auswirkungen auf CD und verwandte Erkrankungen.

## **Ziel 2: Behandlung und Management von Kindern mit CD**

Die Identifizierung und Erprobung möglicher Präventionsstrategien in der Praxis ist ein langwieriger Prozess, der der derzeitigen Patientenpopulation mit CD nicht direkt zugutekommt. Daher ist es sehr wichtig, die Behandlung der derzeitigen Patienten zu verbessern. Die Forschungsbemühungen in dieser Arbeit reichen daher von der Untersuchung veränderbarer Risikofaktoren für CD bis zur Verbesserung des CD-Managements, wobei der Schwerpunkt auf der Rolle von Ernährungsfaktoren liegt. In dieser Hinsicht bestand das zweite Ziel dieser Arbeit darin, die Herausforderungen der GFD über die Gluten Eliminierung hinaus zu erforschen (Kapitel 4 bis 6). Bei den wissenschaftlich fundierten Strategien für die Nachsorge von pädiatrischer CD gibt es eine erhebliche Wissenslücke. Insbesondere der optimale Zeitpunkt und die Häufigkeit serologischer Tests sind nach wie vor unklar. Für Faktoren, die zusätzlich zu den CD-Autoantikörpern durch serologische Tests bewertet werden können, wie z. B. Mikronährstoffwerte, Blutbild, Schilddrüsenhormonspiegel und Leberfunktionstests, gibt es nur unzureichende Erkenntnisse über die Häufigkeit von Anomalien. Darüber hinaus ist das Verständnis der klinischen Bedeutung kurz- und langfristiger Anomalien dieser Parameter bei Kindern mit CD begrenzt.

Die Literaturübersicht in dieser Arbeit (Kapitel 4) untersuchte die vorhandenen Belege für das Auftreten von Nährstoffmängeln während der Nachsorge von CD bei Erwachsenen und Kindern. Darüber hinaus haben wir die Zufuhr verschiedener Vitamine und Mikronährstoffe bei CD-Patienten nach einer GFD untersucht. Sowohl diese Überprüfung als auch die retrospektive pädiatrische CD-Kohortenstudie am Maastricht UMC+ erbrachten den Nachweis, dass neu diagnostizierte Patienten und Patienten nach einer GFD häufig Nährstoffdefizite aufweisen, insbesondere im Hinblick auf Vitamin-D-, Eisen- und Ferritinmangel (**Kapitel 4 und 5**). Diese können auch nach langfristiger Befolgung einer GFD auftreten (Kapitel 5).

Darüber hinaus haben die in den Kapiteln 4 und 5 vorgestellten Ergebnisse gezeigt, dass CDD ein Risikofaktor für die Entwicklung eines Nährstoffmangels aufgrund einer unzureichenden Nahrungsaufnahme sein kann. Dies unterstreicht auch die Bedeutung einer angemessenen Ernährungsberatung und -aufklärung von CD-Patienten und ihren Familien, wobei der Schwerpunkt nicht nur auf der ordnungsgemäßen Eliminierung von Gluten aus der Ernährung liegt, sondern auch auf der Gewährleistung einer angemessenen Aufnahme anderer Nährstoffe. In

der Literaturübersicht haben wir auch gezeigt, wie schwierig eine angemessene Nährstoffzufuhr nach einer CDI ist.

Dementsprechend zeigte die während der COVID-19-Pandemie durchgeführte Fragebogenerhebung auch andere Aspekte der GFD, die weiterer Aufmerksamkeit bedürfen (Kapitel 6). Es wurde festgestellt, dass Essen im Freien die Hauptquelle für die unbeabsichtigte Aufnahme von Gluten ist, was zu erhöhter Angst und einer Veränderung des Sozialverhaltens führen kann. Als positives Nebenprodukt der COVID-19-Pandemie berichteten Patienten und ihre Eltern über die Vorteile des Online-Einkaufs von glutenfreien Produkten (Kapitel 6). Die Herausforderungen, die das Leben mit CD und die Befolgung einer GFD mit sich bringen, sind im täglichen Leben von CD-Patienten, ihren Familien und ihren Betreuern nach wie vor präsent.

### **Fazit: Einsatz der Ernährung in der Prävention und Behandlung von CD**

Diese Arbeit zeigt, dass es wichtig ist, sich auf die Ernährung im weiteren Sinne zu konzentrieren und nicht nur Gluten wegzulassen, und dass dies die Gesamtstrategie zur Verringerung des Risikos von CD verbessern kann. Um die Bedeutung von Nährstoffmängeln im Zusammenhang mit CD zu beurteilen, müssen die potenziellen klinischen Folgen in dieser speziellen Patientengruppe weiter untersucht werden. Die Klärung dieser Aspekte könnte zu einem neuen Verständnis der Bedeutung des Ernährungszustands in dieser speziellen Patientengruppe im Vergleich zur Allgemeinbevölkerung führen. Diese Informationen könnten dann genutzt werden, um einen Konsens über die Notwendigkeit und Häufigkeit der Überwachung des Ernährungszustands bei Kindern mit CD nach GFD als Teil ihres klinischen Managements zu erreichen. Da die Häufigkeit der verschiedenen Mangelzustände sehr unterschiedlich ist, könnte erwogen werden, die Nährstoffe nach der Häufigkeit ihres Mangels zu kategorisieren und die Überwachung entsprechend zu planen. Gleichzeitig ist es wichtig, die gemeinsame Verantwortung von Gesundheitsdienstleistern, der Regierung und der Ernährungsindustrie für den Erfolg der Patienten anzuerkennen. Daher ist es von entscheidender Bedeutung, dass alle Beteiligten ihre Verantwortung für die Aufrechterhaltung der Ernährungsqualität der GFD wahrnehmen.

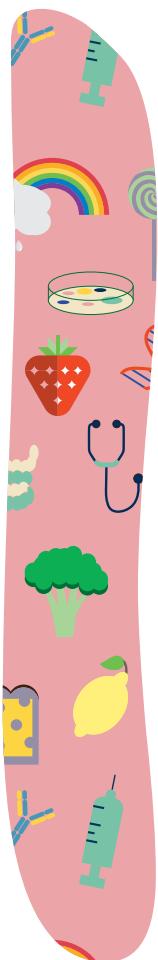
Die Ernährung spielt eine wichtige Rolle bei Gesundheit und Krankheit. Das Potenzial von Ernährungsfaktoren als Risikofaktoren für den Ausbruch oder das Fortschreiten von Krankheiten ist vor allem bei nicht übertragbaren Krankheiten bekannt, z. B. beim Fett- und Zuckerkonsum im Zusammenhang mit Typ-2-Diabetes und Lebererkrankungen, aber auch bei chronisch entzündlichen Krankheiten wie

der chronisch entzündlichen Darmerkrankung (IBD). Dabei kann die Ernährung auch als Präventions- oder Behandlungsstrategie eingesetzt werden. Ein hervorragendes Beispiel ist die GFD für die Behandlung von CD.

Es ist weithin anerkannt, dass Ernährungsfaktoren, insbesondere die Gluten Belastung, einen wesentlichen Beitrag zur Entstehung von CD leisten. Darüber hinaus wird vermutet, dass eine westliche Ernährung zur Entstehung von CD beitragen kann (**Kapitel 3**). Daher ist die Ernährungsberatung ein vielversprechendes Ziel für die Prävention von CD, da die Bestandteile der westlichen Ernährung als Risikofaktoren für die Entwicklung von CD gelten. Darüber hinaus sollte die künftige Forschung auf weitere Bestandteile der „westlichen Ernährung“ einzeln und in Kombination ausgedehnt werden. Es ist wahrscheinlich zu simpel, die Wirkung einer Komponente auf die Integrität der Schranke einzeln zu betrachten, denn ihre Kombination kann tatsächlich zu veränderten Wirkungen führen, wie in dieser Arbeit gezeigt wurde.

In Zukunft müssen wir weiter erforschen, wie wir die Kraft der Ernährungsfaktoren zum Nutzen der Krankheitsprävention und eines besseren Krankheitsmanagements nutzen können.





IMPACT

## **Impact**

Celiac disease (CD) is an auto-immune condition triggered by gluten consumption, causing intestinal damage and various health issues, such as failure to thrive, abdominal complaints and impaired bone health. Notably, the incidence of auto-immune disorders is increasing in all age groups. The related growing prevalence of CD, affecting approximately 1-2% of the population, and its chronic nature underscore the high economic and societal impact of CD. One notable distinction between CD in adults and children is the clinical presentation, and naturally, the duration of the chronic disease. The burden of CD in children is multifaceted, encompassing physical health due to nutrient deficiencies and a broader societal and psychological burden, especially from following a strict gluten-free diet (GFD) (1). Adhering to a GFD can impose a considerable financial burden on CD patients and their families. Additionally, there is also a substantial financial burden on society. Patients with CD in the US for instance experience substantially higher healthcare expenses compared to control groups matched for comorbidities and demographic factors (2). The mean annual all-cause healthcare costs of CD patients are comparable to those of patients with Crohn's disease in the US and were increased by \$3776 two years after diagnosis of CD compared to matched controls (2). Consequently, the prevention of CD represents a paramount approach for alleviating this financial strain.

The exact reasons for the rising incidence of CD in children remain unclear, and the role of environmental, psychological, and societal factors contributing to this trend need further studying. The overarching goal of this thesis was to reduce the disease burden of CD in children by focusing on disease prevention on the one hand and improving treatment of patients with CD on the other hand. Thereby, this thesis specifically addresses the challenges of CD in children and underscores the necessity for a focus on the pediatric population to enhance the management and quality of life for children affected by CD.

The findings from the studies in the current thesis can provide leads, not only for scientists, healthcare providers, educators, and policymakers, but also for patients with CD and their families, offering pathways towards improved strategies for managing CD and disease prevention. The socioeconomic, seasonal, and regional disparities highlighted in the incidence of auto-immune diseases, including CD, provide indications for potentially modifiable factors in the risk of developing CD. The emphasis on prevention is paramount, and the identification of new targets and leads is crucial, not only for CD but for other intestinal diseases characterized by the disruption of barrier function. Herein, focusing on potential factors that can be targeted and harnessed in disease prevention will yield a greater impact than merely understanding disease pathophysiology. Dietary factors represent a robust

example of a potentially modifiable risk factor associated with CD. The first aim of this thesis was to investigate the role of dietary factors and barrier disruption in CD etiology.

We found, that intestinal damage could be a contributing risk factor to CD onset, as we identified different patterns in a biomarker for intestinal damage in a subgroup of children that developed CD, compared to a group that did not develop CD. Next we aimed to investigate the role of the diet on intestinal permeability in an *in vitro* cell model. We found that dietary factors associated with an increased CD risk, namely gliadin, glucose and fructose dose-dependently can lead to an increase in small intestinal permeability. Gliadin is the part of gluten, that triggers the autoimmune reaction in CD, therefore called the immunogenic component of gluten. This provides evidence for the hypothesis that high exposure to these dietary factors early in life could contribute to increased intestinal permeability, resulting in the passage of gliadin across the intestinal wall. This would enhance the contact of gliadin to the immune system and increases the risk of the CD-specific immune reaction. Surprisingly, in our study of the effects of these sugars and gliadin on intestinal permeability, we found an altered effect when exposing the cell model to the components separately as compared to a combined exposure. This underlines, that future research should focus on studying dietary factors in realistic approximations of a meal and more physiological conditions.

The findings presented in this thesis show interesting tethering points and merit for future research focusing on nutrition, especially the impact of the Western diet and intestinal barrier damage and dysfunction as part of CD etiology.

At present, the only treatment available is GFD. Maintaining a GFD presents distinct challenges for children with CD. Key issues include the risk of nutrient deficiencies due to restricted food choices, social hurdles in environments like school lunches, and the psychological burden of constant dietary vigilance. The COVID-19 pandemic may have intensified these challenges, disrupting routines and access to gluten-free products. These factors underscore the complexity of adhering to a GFD for young patients with CD. The second aim of this thesis was to evaluate the challenges of the GFD, beyond the elimination of gluten. We evaluated the risk to develop nutrient deficiencies while following a GFD, in a review of the current literature as well as our own cohort of children with CD. Both studies showed that nutrient deficiencies such as iron and vitamin D deficiency occur frequently even after following a strict GFD for a prolonged time. Although we found these common deficiencies in our own study as well as in the literature, the full extent of the problem is still unclear. This is mainly because possible clinical relevance and implications on short-term and especially in the long-term are still not known. In our literature review we found for instance, that impaired bone

health is an important long-term consequence of CD, even when treated correctly with a GFD. The known increased risk for bone fractures and osteoporosis of patients with CD at older age could be exacerbated by (re-) occurring periods of vitamin D deficiency. A questionnaire study we conducted during the second lockdown period of the COVID-19 pandemic in the Netherlands revealed additional challenges patients and their parents are facing, which reach beyond the pandemic. Examples are the unintentional exposure to gluten when eating outdoors and the lack of knowledge on availability of gluten-free products. Our findings on the management of CD through diet and the risk to develop nutrient deficiencies, highlight the need for better patient education regarding the GFD. The evidence presented here, combined with the existing literature, can contribute to a more evidence-based approach to CD management, aiding patient, healthcare providers and other stakeholders to enhance the health and overall wellbeing of individuals with CD.

Improved education and enhanced collaboration with dietitians, General Practitioners, and all healthcare providers are pivotal in improving care for patients with CD. Herein, results such as shown in this thesis show, that dietary education must extend further than only the elimination of gluten from the diet. Examples for stakeholders outside of direct healthcare providers can be schools, but also restaurants and other establishments providing food, the food industry as well as authorities regulating them. Improvements can be made to make eating outside of the home feel safe for all patients with CD or other dietary restrictions, through good education of food workers, effective regulations and safeguarding and standardizing of appropriate food safety labels (3). This includes improvement of education and regulation concerning cross contamination leading to small traces of gluten that can trigger disease, but as important, also on the nutritional quality of gluten-free products and meals.

Working towards more evidence-based guidelines on the management and follow-up of CD for clinicians, can additionally give a basis for a part of the substantial health care costs of CD follow-up. Evidence-based decision making on for example frequency and nature of serological tests can potentially prevent unnecessary health care costs next to lowering the burden of disease follow up for patients themselves.

Addressing the broader impact of this research, it must be noted, that most research conducted so far predominantly pertains to Western society, including mainly European and Caucasian demographics. To truly understand the multifaceted nature of CD and its risk factors, incorporating a more diverse ethnic and cultural background in research is crucial. This diversity not only enriches the research but also broadens the impact of the study outcomes. This is an important limitation of

the work presented in this thesis as well as in CD research in general. For example, the disease burden is hypothesized to be substantial in Africa, where there are almost no publications on CD (4, 5). The distribution of HLA risk groups in Ethiopia is similar to Sweden, yet the prevalence of CD is not even known in large parts of the world, including Ethiopia and some of the most populous countries like China, Indonesia, Pakistan, Nigeria and Bangladesh.

It is important that research findings are widely shared with various stakeholders, e.g. explicitly including patients and their environments. In order to broaden the impact of this thesis, the studies have been presented at various national and international conferences, such as ESPGHAN, NUTRIM days, and the NCV dag of the Dutch patient society for CD, to peers, the lay public, and patients themselves. The Studium Generale lecture at Maastricht University served as a platform to articulate the findings and implications of the studies included in this thesis to a diverse audience, fostering awareness and understanding of CD and its multifaceted implications. Moreover, the commitment to open access publication ensures that the forthcoming papers of this thesis are accessible to a wide array of individuals and groups, facilitating the dissemination of knowledge and fostering an inclusive academic environment.

In conclusion, the work in this thesis contributes to the ongoing dialogue on CD, offering insights that aid significant steps forward in understanding and managing the disease. The need for more inclusive and diverse research is evident, and the contribution of this work to the global conversation on CD is a reminder of the interconnectedness of our world and the shared responsibility to enhance knowledge and understanding for the betterment of all.

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## About the author

Johanna Monika Kreutz was born in Bonn, Germany. After living in Boston, Massachusetts, USA for four years, she grew up in Berlin, Germany. After finishing her Abitur in 2009, she studied Biomedical Sciences at Maastricht University and received her Bachelor degree in 2012. Johanna completed the Master program Physician Clinical Investigator at Maastricht University in 2017, which included a research internship at the Altonaer Kinderkrankenhaus in Hamburg, Germany and her master internship at the department of pediatrics at Maastricht University Medical Center + (MUMC+).

She worked as a medical doctor for the gastroenterology division of the department of pediatrics for one year, during which she set up her research project next to providing medical care. She applied for and received the competitive NUTRIM graduate program grant from the NUTRIM research school of Maastricht University. This enabled her to start her self-designed PhD project focusing on celiac disease in children in September of 2018 at the department of pediatrics and the department of Internal Medicine, division Gastroenterology- Hepatology at MUMC+.

During her PhD-trajectory, Johanna was an active participant of the international PreventCD study group, attending and presenting during their annual meetings. She gave several oral presentations during national and international conferences and received the Young Investigators award of the ESPGHAN society, enabling her to visit the ESPGHAN Summer School in Basics and Translational Research in Cambridge, UK. She was a board member of the Pélerin Symposium committee and the National Childhood Obesity symposium committee. Further she participated in several events organized by the national patient organization for celiac disease, NCV, where she gave oral presentations, workshops for children and was a panelist in central discussions.

Next to her research activities, Johanna set up a specialized consultation for pediatric patients with celiac disease at the outpatient clinic of the department of pediatrics of the MUMC+, now MosaKids Children's Hospital, where she provided patient care.

After completing her PhD thesis in December 2023, she resumed her clinical work in March 2024 at the department of pediatrics in the Zuyderland hospital in Heerlen, The Netherlands.

## List of publications

Kreutz JM, Adriaanse MPM, van der Ploeg EMC, Vreugdenhil ACE. Narrative Review: Nutrient Deficiencies in Adults and Children with Treated and Untreated Celiac Disease. *Nutrients*. 2020 Feb 15. doi: 10.3390/nu12020500.

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## List of abbreviations

Anti EMA	Anti-endomysium antibodies
Anti tTG	Anti-tissue transglutaminase
BMD	Bone mineral density
BMI	Body mass index
CD	Celiac Disease
CV	Coefficient of variation
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-linked immunosorbent assay
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
FITC-4D	Fluorescein isothiocyanate–dextran
GFD	Gluten-Free Diet
GLUT5	Glucose transporter 5
GPTC	Gliadin digested with pepsin, trypsin, and chymotrypsin
GPT	Gliadin digested with pepsin and trypsin
Hb	Hemoglobin
IBD	Inflammatory Bowel Disease
IDA	Iron deficiency anemia
IEL	Intraepithelial lymphocytes
I-FABP	Intestinal fatty-acid-binding protein
LDH	Lactate dehydrogenase
MASLD	metabolic dysfunction-associated steatotic liver disease
MTT assay	Methylthiazolyldiphenyl-tetrazolium bromide cell viability assay
MUMC+	Maastricht University Medical Centre
NAFLD	Non-alcoholic fatty liver disease
NHANES	National Health and Nutrition Examination Survey
SGLT1	Sodium-glucose co-transporter 1
TEER	Transepithelial electrical resistance
ZO-1	Zonula occludens-1





