The role of gluten challenge in the diagnosis of celiac disease: a review

Alina Popp[a,b*], Pilvi Laurikka[b,c*], Diana Czika[a], Kalle Kurppa[b,d,e]

[a]University of Medicine and Pharmacy Carol Davila and National Institute for Mother and Child Health, Bucharest, Romania

[b]Celiac Disease Research Center, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland

[c]Department of Internal Medicine, Tampere University Hospital, Tampere, Finland

[d]Tampere Center for Child, Adolescent and Maternal Health Research, Tampere University and Tampere University Hospital, Tampere, Finland

[e]The University Consortium of Seinäjoki, Seinäjoki, Finland

[*]Shared first authorship

Correspondence and contact: Kalle Kurppa, MD, PhD, Faculty of Medicine and Health Technology, Tampere University Hospital, Tampere Finland. Email: kalle.kurppa@tuni.fi

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Abstract

Introduction: Duodenal biopsy is the gold standard in the diagnosis of celiac disease, with increasing utilization of serology. A gluten challenge may be required, for example, when dietary gluten reduction precedes appropriate diagnostic evaluations. Evidence on the best challenge protocol is currently sparse. Pharmaceutical trials in recent years may have provided new insights into the challenge and advanced the development of novel sensitive histological and immunological methods.

Areas covered: This review outlines the current perspectives on the use of gluten challenge in the diagnosis of celiac disease and explores future directions in this area.

Expert opinion: Comprehensive elimination of celiac disease before dietary gluten restriction is essential to avoid diagnostic uncertainties. Gluten challenge continues to have an important role in certain clinical scenarios, although it is important to understand its limitations in the diagnostic evaluation. The evidence so far permits no unequivocal recommendation considering the timing, duration, and amount of gluten used in the challenge. Thus, these decisions should be made on a case-by-case basis. Further studies with more standardized protocols and outcome measures are called for. In the future novel immunological methods may help to shorten or even avoid gluten challenge.

Keywords: Celiac disease; gluten; challenge; children; diagnosis; tetramers; drug

Article highlights:

- It is essential to perform comprehensive diagnostic evaluations for celiac disease before introducing any dietary gluten restrictions.
- Gluten challenge continues to have an important role in situations in which a gluten-free diet has already been initiated before appropriate investigations or when the celiac disease diagnosis is otherwise inconclusive.
- Due to limited scientific evidence, the timing, duration, and amount of gluten used in the challenge should be determined according to individual assessment and joint decision-making.
- It is important for clinicians to recognize that there are still significant limitations in the utilization and interpretation of the gluten challenge.
- More studies with standardized protocols and outcome measures are warranted to determine best practices in the implementation of the challenge.
- Recent pharmaceutical studies have driven the development of more sensitive immunological methods and other outcome measures that may help to shorten or even eliminate the need for gluten challenge.

1. Introduction

Celiac disease (CeD) is an immune-mediated disease driven by dietary gluten and characterized by inflammation of and morphological damage to the small-bowel mucosa [1]. Histopathologic examination of the mucosa utilizing endoscopically obtained biopsies remains the gold standard for diagnostic outcome, although the high precision of modern CeD serology has promoted an ongoing shift towards less invasive approaches [2-4].

Gluten challenge (GC) is utilized in clinical practice when a patient with CeD suspicion has reduced gluten intake before adequate diagnostic evaluations and needs to be re-exposed for a variable period of time. GC was previously a mandatory part of the CeD diagnosis in the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria [5, 6]. The complex protocol comprised demonstration of duodenal lesion in initial investigations, followed by histological recovery on a gluten-free diet (GFD), and a subsequent relapse after gluten re-introduction. A so-called "2-year rule" was applied, based on the assumption that detectable relapse would be present after two years on gluten [6]. Eventually the requirement of GC was dropped from the criteria [7] but still plays an important role in diagnostically challenging clinical scenarios.

The development of serological assays has led to major changes in the pediatric criteria and the current ESPGHAN guidelines allow no-biopsy diagnosis in children with tissue transglutaminase antibodies (TGA) >10x upper limit of normal (ULN) and positive endomysial antibodies (EMA) [2]. In adults, biopsy remains mandatory in most countries, although there is an increasing debate as to whether the serology-based criteria could also be adopted to this age group [3, 4, 8, 9]. Nevertheless, biopsy also remains obligatory in children with low (<10x ULN) positive TGA [2]. These individuals are often also the diagnostically most challenging cases, who may need repeated

evaluations and, in many cases, also GC. Although the present diagnostic criteria for children and adults are not identical, the histopathological and serological assessments used in diagnostically challenging cases are not markedly age-dependent.

Histology remains the gold standard outcome not only for the diagnosis but also for the evaluation of treatment response. In fact, its role may even be increasing due to the recent emergence of drug trials driven by challenges with GFD [10-13]. Of note, the roles of GC and histology differ somewhat between trials and diagnostic evaluations as the former usually involves only a short-term challenge and non-diagnostic mucosal changes could suffice.

In this review, we outline the current perspectives on the use of GC in the routine diagnosis of CeD and also review what has been learned from recent pharmaceutical trials. We also briefly discuss possible future directions. The focus will be on studies conducted in the era of modern serology in the twenty-first century.

2. Indications for gluten-challenge in clinical practice

There are several reasons why well-planned GC may play an important role in the diagnosis of CeD. One particularly common problem is short duration of use or reduced dietary gluten intake before appropriate diagnostic evaluations. This may be due, for example, to young age of the child or to presence of CeD in another family member resulting in the use of gluten-free products in (shared) family meals. Furthermore, GFD is nowadays often tried, for instance, for clinical symptoms reminiscent of irritable bowel syndrome or because of other anticipated health benefit such as possible prevention of another autoimmune condition [14-17]. It must be emphasized that clinical response to GFD is not specific to CeD and that the diagnosis should be based on objective findings in serology and histology while the patient adheres to a gluten-containing diet.

Even if gluten has been regularly ingested prior to the investigations, there may be challenges in the diagnostic process possibly resulting in inconclusive results. For example, asymptomatic or only mildly symptomatic patients detected by at-risk group screening may decline to undergo the endoscopic investigations. In addition, the accuracy of the widely used graded histological analysis is suboptimal in cases with less severe mucosal damage [18-21] and, on the other hand, not even an advanced lesion is pathognomonic for CeD [22, 23]. Serology could be helpful in these situations, but the lack of standardization of the TGA tests is an additional challenge [24]. Moreover, adults in particular may present with a true seronegative CeD [25].

In these circumstances, major efforts should be made to eliminate CeD before starting the GFD. Seronegative subjects in particular benefit from the identification of CeD-associated HLA alleles, the lack of which makes CeD unlikely [2, 26-28]. There are also certain more sophisticated histological methods, such as determination of intestinal TGA-specific IgA deposits, which have shown excellent specificity for CeD even in seronegative patients [2, 29-31]. However, these methods have not so far been widely adopted in clinical practice and in most cases a GC may be the only way to further specify an inconclusive diagnosis.

3. Pre-challenge evaluations

The planning of the GC can be started after the aforesaid diagnostic considerations. Meticulous planning and joint decision-making are critical for a successful GC in all age groups. Although mostly similar, children differ somewhat from adult patients regarding the pre-challenge evaluations. Acceptance of the GC may vary depending on the child's age and the previous experiences of diagnostic investigations of both child and parents, as well as on the degree of family anxiety regarding the ingestion of a potentially harmful gluten.

In all patients, the preceding evaluations should involve assessment of possible symptoms and clinical findings and previous use of gluten as well as measurement of CeD serology and other relevant laboratory parameters. Availability of precise growth data is essential in children. The need for histological assessment before the challenge should be decided on an individual basis considering previous medical history. This is particularly important in pediatric patients, for whom general anesthesia is usually required for the endoscopy. An additional challenge with children is that gluten re-introduction should be avoided during periods of accelerated growth [2, 32].

4. How much gluten and for how long?

The daily ingestion of gluten in a normal diet varies widely, and targeting the amounts used in general population may be unrealistic due to tolerance problems. Regular gluten intake has been estimated to be approximately 10–20 g/day in adults and 5–15 g/day in children [33,34], but more studies from different geographical areas are called for. Particularly in children, there is a scarcity of evidence on how much gluten should be given and for how long to ensure a reliable outcome in GC (Table 1). Therefore, it has been necessary to extrapolate results from adult studies to children.

4.1. Children

In an early study, Korponay-Szabo et al. observed a serological (EMA) and histological relapse in the great majority of pediatric CeD patients challenged with 5-10 g of purified gluten daily for six months [36]. More than 50% of the children already showed positive seroconversion after three months. Additionally, Holm et al. challenged ten children with 7-19 g of daily gluten and reported serological (TGA and EMA) and histological relapse after 3-12 months of surveillance [34] (Table 1). Seroconversion was observed as early as after one month in half of the subjects. In a more

exploratory study, Hardy et al. detected gluten-specific T-cell response within three days on GC in 30 out of 41 CeD children consuming 1-3 slices of gluten-containing (~3-9 g) bread per day [39].

Based on expert opinions and the limited evidence, ESPGHAN recommends starting the GC with 10-15 g of daily gluten [32], the duration of the challenge depending on the response. The determination of serum TGA should start as early as after one month, followed by re-evaluation tri-monthly for up to one year in the absence of clinical or serological relapse [32]. Long-term follow-up on a normal gluten-containing diet with subsequent annual checkup visit is recommended thereafter. It is important to realize that some children may require years or even decades of regular gluten consumption before eventually relapsing [38]. Furthermore, the clinical response to the challenge may change from the original gastrointestinal presentation to extraintestinal symptoms [38].

4.2. Adults

In the adult CeD guidelines, the commonly recommended procedure for diagnostic GC is to provide at least 6-10 g gluten/day for 1-3 months before the determination of CeD serology [9, 26], even though evidence supporting this approach has been limited [40]. In recent years, several prospective trials have aimed at estimating the duration and dosage sufficient to induce significant clinical, serological, and histological changes during the GC (Tables 2 and 3). For example, Leffler et al. suggested that only 3 g of gluten/day for two weeks would suffice to induce morphological changes in the small-bowel mucosa in the majority of CeD patients [49], while Lähdeaho et al. reported doses of 1-5 g be adequate in 67% of subjects after six weeks [60]. These results have

been challenged in more recent studies, in which at least the shorter GC has seldom been sufficient to induce significant lesion [52, 57].

Regarding serology, while the autoantibodies have excellent sensitivity for untreated CeD, they are less successful in recognizing histological damage during a GFD [68] as well as in detecting CeD-related immunological activation during GC. For instance, in the study by Lähdeaho et al. 43% of the patients seroconverted positive during a 12-week GC with daily gluten doses of 1-5 g [60], while in that by Leffler et al. this occurred in 50% of patients after two weeks on 3/7.5 g [49]. In a more recent study, seroconversion occurred in only 16% of the patients after a six-week GC with 3 g [66]. Altogether, the seroresponse seems to vary substantially between adult CeD patients, the current tests being unreliable predictors of histological changes (Tables 2 and 3).

The diverse study protocols complicate the evaluation of symptoms in relation to GC. Accordingly, a recent meta-analysis concludes that response to different gluten doses may be particularly individual and that the existing data have severe limitations due to disparate protocols [69]. There is often a rapid response which may cause discontinuation of the GC, with low daily amounts (1-3 g) likely being better tolerated than moderate doses (3-5 g) [60]. However, the symptoms often abate or at least stabilize when the GC is resumed [67, 70]. Interestingly, vomiting and nausea seem to be particularly gluten-specific symptoms, although this has not been reported in all studies [54, 61, 62, 66, 70, 71]. Altogether, the evolution of symptoms may be affected by many individual factors besides daily gluten dose and duration of the challenge, further limiting the use of clinical response as a reliable outcome measure.

5. Points to consider

Clinical evaluation during the GC should consider the presence of non-gluten ingredients that could trigger the reported symptoms, such as FODMAPs and wheat components other than gluten [72-76]. If possible, these should be excluded, as functional symptoms are common in CeD patients [77]. Particularly sensitive patients could benefit from a multidisciplinary team including a dietician. Systematic recording of the symptoms is mandatory and, although scientific evidence is lacking, with young children the role of caregivers is likely central. CeD-specific questionnaires may further improve the assessment of these patient-related outcome measures but interpretation of the response is often challenging due to symptom heterogeneity.

The results of serology should also be interpreted with caution. Only calibrated and well-validated serological tests should be utilized [2, 21]. Regular gluten consumption is imperative; a markedly irregular challenge with low quantities may not suffice to cause a detectable change in the TGA levels. Altogether, even if correctly applied, as mentioned (Section 4.2), there is major individual variation in the serological response and only a minority, particularly of adult CeD patients, may exhibit positive seroconversion during GC. The unsatisfactory sensitivity is further demonstrated by the poor correlation reported between dietary lapses and TGA levels on GFD [78-80].

It is strongly recommended to offer intestinal biopsy in the case of clinical and/or serological relapse, especially in adults. The decision to embark on the procedure should be discussed with the patient and – in children – the caregivers. Of note, due to disagreement, endoscopy was not included in the ESPGHAN recommendations [32]. The main challenge is again individual heterogeneity and insufficient sensitivity (Chapter 4.2). This problem is further exacerbated by the substantial intra- and interobserver variability in the histologic evaluation [81-84]. Quantitative histomorphometric measurements could offer more replicable readouts [20, 81, 85] and, if accessible, the above-mentioned IgA deposits could be valuable [2, 29-31]. Additional endoscopic

and imaging techniques for the evaluation of the mucosal damage have been introduced but their availability remains limited [86-88].

Of note, if GC is particularly poorly tolerated or is estimated not to be clinically meaningful, one option is to treat the patient applying the same principles as for confirmed CeD. Such a decision should be made only after thorough discussion with the patient, emphasizing the requirement for a strict and lifelong GFD.

6. Future directions

GC continues to pose significant challenges, including tolerability, particularly in view of the long-term challenge and insufficient accuracy of the current readouts with the short-term challenge. Consequently, there is a need for more sensitive biomarkers either shortening the challenge or totally obviating it. This might be achievable using novel sophisticated immunological techniques.

For example, CeD patients are known to have a persistent repertoire of gluten-specific CD4+ T cells in the intestine and peripheral blood [89]. When stimulated by gluten, these cells secrete inflammatory mediators such as interleukin (IL) 2, 8 and 10 [70], the measurement of which may allow early detection of CeD-related immune activation. IL-2 is especially promising as it exhibits a rapid and dose-dependent increase during short-term GC [57, 90]. In fact, the response can be detected as soon as 2-6 hours after a single 6-g bolus [57, 70, 90].

Another promising option for rapid readout in GC is detection of gluten-specific T cells gathered from the peripheral blood samples, for example by culturing the cells together with gliadin peptides and subsequently identifying them with ELISpot assay [91]. Of note, as gluten-specific CD4+ T cells are rarely detected in blood, the use of ELISpot demands a three-day GC in patients on GFD

[92] but additional research is ongoing to improve the methodology [93]. Furthermore, gluten-specific T cells could be labeled among the peripheral blood mononuclear cells with HLA-DQ:gluten tetramers and detected by flow cytometry. Sarna et al. [52] reported 80% of CeD patients to display a ≥100% increase in blood CD4+ effector-memory gut-homing HLA-DQ:gluten tetramer-binding T cells after six-day GC, the method having significantly better sensitivity than biopsy at day 14. As an additional benefit, the tetramers could enable the recognition of gluten-specific T cells even without the GC [94, 95]. Nevertheless, the procedure is demanding and requires a considerable volume of blood [91].

7. Conclusions

The diagnosis of CeD is usually quite straightforward, but a well-planned GC continues to play an important role. At present, however, there is a major shortage of scientific evidence in this area, and additional prospective studies with standardized study protocols and outcome measures are called for. A more standardized use of GC in scientific research would likely help to reach a consensus on the best GC protocol in clinical practice, too. It is imperative for clinicians to understand the limitations of the current GC procedures in diagnostic evaluation. In particular, the need for a challenge should be reduced by meticulously investigating the possibility of CeD before any dietary gluten reduction takes place. Encouragingly, the development of more sensitive histological and other outcome measures is actively ongoing. Recent pharmaceutical studies have also accelerated the development of novel immunological biomarkers, which in the future may make it possible to totally dispense with the challenge.

8. Expert opinion

In the last decade, the improved availability of commercial gluten-free products has considerably facilitated maintaining a GFD for treating CeD. As a downside, it has become increasingly common to initiate at least partial gluten restriction without appropriate exclusion of CeD, for example due to the presence of the condition in a relative, because of suspected functional gastrointestinal symptoms or in hope of other health benefits. This may constitute a major challenge for subsequent diagnostic evaluations. Unfortunately, in light of` the existing evidence, it can be extremely challenging or sometimes even impossible to set the diagnosis after a long-term GFD. Missing the correct diagnosis may expose the patient to various CeD-associated severe complications, particularly as individuals with self-initiated GFD may not have received appropriate guidance from a professional dietician and perceived the necessity of a strict dietary treatment. Therefore it is strongly recommended to eliminate the possibility of CeD before any dietary gluten reduction. Besides educating healthcare professionals, this issue should also be advanced by disseminating information about the diverse clinical manifestations of CeD among the general population.

There may also be various technical pitfalls in the diagnostic investigations regardless of previous gluten consumption or other patient-related factors. As regards serology, it is important that only well-calibrated and validated TGA tests are utilized to ensure reliable case-finding and screening of CeD. This is as important as ever, given the increasing amount of data supporting the expansion of serological diagnostic criteria for CeD, also for adults. When the diagnosis is based on histopathologic analysis of the duodenal biopsy, correct orientation, and assessment of the mucosal samples by an experienced pathologist is particularly important to avoid misdiagnosis. Aiming at high-quality first-line diagnostics also alleviates possible doubts either on the part of the patient and/or the physician and may decrease the risk of later dietary transgressions.

Despite the aforesaid problems, GC continues to have an important role when the diagnosis of CeD remains unsure. Pharmaceutical studies conducted in recent years have provided valuable information about the performance of different serological and histological outcome measures in GC. However, in many respects the current evidence still does not suffice to provide explicit recommendations on the optimal implementation of the challenge. A particular obstacle is that, due to the lack of consensus regarding the most suitable protocol, the trials hitherto conducted have varied markedly regarding the form and dosage of gluten used in the GC and its duration, as well as the study endpoints. Consequently, there is an urgent need for additional research with more standardized challenge protocols and outcomes. An additional challenge is the very limited number of pediatric GC studies.

Patients' frequently poor tolerance of GC because of symptoms and/or fear of gluten-induced complications of CeD may significantly limit the use of long-term challenge for diagnostic and research purposes. On the other hand, a short-term GC appears to exhibit insufficient sensitivity when applying traditional serological and histological outcomes. Besides, with improved imaging and other tools, better results could also be achieved by measuring CeD-specific immunological activation. In this regard, the recently introduced innovative approaches, such as HLA-DQ:gluten tetramer blood test and sequential measurement of IL-2, reinforce the belief that in the near future it may be possible to identify CeD reliably even after a very short and practical GC.

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ORCID

Kalle Kurppa http://orcid.org/0000-0003-4757-2164

Pilvi Laurikka https://orcid.org/0000-0002-3365-3257

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Table 1. Gluten challenge studies with duration of over 14 days executed in pediatric celiac disease (CeD) patients.

Study	Study type	Participants	Objectives and outcomes	Main results
Mäki et al. 1989 (Finland) [35]	Open trial with ≥10g daily gluten. Follow-up visits at 1 month and thereafter quarterly. Biopsy after clinical or serological relapse and after 2 years.	29	To assess the optimal follow-up strategy for post-pubertal GC. Symptoms, histology and serology (AGA, ARA).	Mean time to ARA-positivity 0.6 years, histological relapse was seen in all patients with positive ARA. Four subject (11%) did not relapse within 2 years.
Korponay-Szabó et al. 1997 (Hungary) [36]	Open trial with 5-10 g daily gluten daily until relapse. Follow-up visits every 3-6 weeks up to 9 months. Biopsy after clinical or serological relapse and after 2 years.	153	To study the specificity of EMA (titer 1: >80) for CeD during GC. Ninety patients (group A) initially positive and 7 negative (B) for EMA; no result in 56 patients (C).	Histological relapse seen in 100% (A), 14.3% (B), and 76.8% (C). EMA-positivity at 3 months in 65.7% and at 6 months in 89.9%. All subjects with histological relapse showed EMA-positivity, whereas non-CeD patients remained consistently EMA-negative.
Holm et al. 2006 (Finland) [34]	Open-label RCT with 14 g daily gluten until histological relapse. Biopsies at 1, 6, 24 months, clinical follow-up of 7 years.	10	To evaluate changes in serology and small-bowel mucosal histology during GC and oats-containing GFD.	On GC, EMA and TGA became positive within a month in 5 patients and in all at histological relapse. Significant decrease of mucosal VH:CrD and increase in IELs within 3–12 months.
Koskinen et al. 2009 (Finland) [37]	Open-label RCT, a part of the Holm et al. 2006 study above	10	To investigate changes in TG-targeted IgA deposits during GC and oats-containing GFD.	The intensity of IgA deposits increased parallel with the development of mucosal damage during GC. At the time of relapse all had positive deposits.
Kurppa et al. 2008 (Finland) [38]	Three patients who had confirmed CeD diagnosis in childhood and started a gluten-containing diet after initial remission.	3	To report 1) the sometimes extremely long delay of clinical response and 2) change of CeD phenotype during long-term GC.	1. After 16 years on gluten-containing diet diagnosed with DH. 2. At age 31 years diagnosed with DH after years of incomplete GFD. 3. At 34 y old diagnosed with DH and partial villous atrophy after years of incomplete GFD.

AGA, anti-gliadin antibodies; ARA, anti-reticulin antibodies; DH, dermatitis herpetiformis; EMA, endomysium antibodies; GC, gluten challenge; GFD, gluten-free diet; IELs, intraepithelial lymphocytes; IgA, immunoglobulin A; RCT, randomized clinical trial; TGA, transglutaminase antibodies; TG, transglutaminase; VH:CrD, villous height-crypt depth ratio

Table 2. Gluten challenge studies with duration of ≤14 days conducted in adult celiac disease (CeD) patients

Study	Study design	Participants	Objectives and outcomes	Main results
Cornell et al. 2005 (Australia) [41]	Double-blind, placebo-controlled crossover RCT with 13 g of gluten daily for 2 weeks. Symptoms recorded for 12 weeks.	21	Changes in symptoms, stool consistency, serology or histopathology (taken from 6 patients before and after challenge).	Moderate to severe symptoms in 8 and increase of TGA in 5 subjects, with no significant correlation with these. No significant change in Marsh scores, but only 1 subject had normal duodenal mucosa at baseline.
Tye-Din et al. 2010 (Australia) [42]	Open trial with 150 g barley daily for 3 days. PBMCs collected at day 6.	21	To identify the toxic hordein fractions and to compare the activation of T-cells.	T cells responded in a dose-dependent manner. All hordein fractions were immunotoxic, but this was reduced ~20-fold without the most toxic fractions.
Brottveit et al. 2011 (Norway) [43]	Open trial with 4 slices of gluten-containing bread daily for 3 days. Treated patients, patients with uncertain CeD[1] and non-CeD controls.	13	To compare HLA-DQ:gluten tetramer test and histological responses, and to assess the usefulness of the tetramer test as a diagnostic tool in CeD.	Only 4/13 of the treated CeD patients had changes in Marsh scores after challenge, whereas positive tetramer test was seen in 11/13.
Daveson et al. 2011 (Australia) [44]	Double-blinded, placebo-controlled RCT with 16 g of gluten daily for 5 days.	10	Changes in histology, systemic inflammatory immune responses and symptoms.	Decrease in Marsh score and VH:CrD and increase in CD3+ and CD8+ IELs and IFN-γ -producing T cells. There was abdominal discomfort 1-2 hours after first dose, which resolved within hours.
Beitnes et al. 2012 (Norway) [45]	Open trial with 4 slices of gluten-containing bread daily for 3 days. Controls HLA-DQ2+ subjects on a GFD.	12	To evaluate whether the changes seen in the specific APCs are caused by chronic inflammation, or if they represent early response to gluten.	Increase in CD14+CD11c+ DCs and decrease in CD103+CD11c+ DCs and CD163+CD11c− macrophages was seen in CeD patients. No significant change in histology.
Camarca et al. 2012 (Italy) [46]	Open trial with 12 g gluten daily for 3 days, repeated after 3-10 months on GFD.	14/13	Reproducibility and usability of 3-day gluten challenge.	Gliadin-specific IFN-γ-secreting CD4+ T cells increase from baseline to day 6 after both challenges, with major individual variation.

Leffler et al. 2012 (USA) [47]	Double-blind, placebo-controlled RCT with 2.4 g gluten daily for 14 days. Follow-up up to day 21.	14	Changes in the intestinal permeability, serology, symptoms and quality of life.	Gastrointestinal symptoms increased, but no significant changes in lactulose-mannitol ratio, TGA and quality of life.
Brottveit et al. 2013 (Norway) [48]	Open trial with 4 slices of gluten-containing bread daily for 3 days. Controls DQ2+ non-CeD subjects and uncertain CeD patients.	15	Early mucosal immunological events as measured by cytokine mRNA and immunohistochemistry.	Signs of concomitant innate (IL-8 and MxA for IFN-α) and adaptive (IFN-γ-related genes and TNF-α) immune response.
Leffler et al. 2013 (USA) [49]	Open RCT with 3 g or 7.5 g gluten daily for 14 days. Follow-up up to day 28.	20	Kinetics of histological, serological, and symptomatic responses, and changes of intestinal permeability	Significant decrease in VH:CrD and increase in CD3+ IELs, positive seroconversion in 65%. Gastrointestinal symptoms increased by day 3 but returned to baseline by day 28. No change in lactulose-mannitol ratio.
Tack et al. 2013 (Netherlands) [50]	Double-blind placebo-controlled RCT with 7 g of gluten daily for 2 weeks[2].	7	Changes in serology, histopathology, quality of life and immunophenotype of lymphocytes.	No change in quality of life, EMA or TGA or immunophenotype of lymphocytes, but intensity of transglutaminase 2 targeted IgA deposits increased in 4/7 patients.
Lopez-Palacios et al. 2018 (Spain) [51]	Open trial with 10-14 g gluten daily for 3 days. Non-CeD control group.	15	The utility of detecting activated γδ+ and CD8+ T cells expressing gut-homing receptors in diagnosing CeD during a GFD.	Both γδ+ and CD8+ T cells co-expressed CD103, β7hi and CD38 in every patient with CeD and in one control on day six. IFN-γ and IP-10 were detected after challenge with ELISpot in patients with CeD, but not in controls.
Sarna et al. 2018 (Norway) [52]	Open trial with 5.7 g gluten daily for 14 days.	19	Sensitivity of HLA-DQ:gluten tetramer test and IL-8 compared with histology in detecting gluten-specific immune activation after a short challenge.	Significant reduction of VH:CrD and increase in IELs in 9/19 subjects. IL-8 increased by >100% after 4 hours in 7/19 subjects and tetramer-binding T cells on day 6 in 12/15 subjects.
Tye-Din et al. 2019 (Australia) [53]	Open trial with single 6 g gluten bolus. Non-CeD control group.	25	Nature and kinetics of serum IL-2 changes and symptoms during challenge.	IL-2 elevated by 4 hours in 92% of CeD patients and correlated with timing and severity of symptoms. No release in controls. Peak in symptoms at 3 hours.

Daveson et al. 2020 (Australia) [54]	Double-blind, sham-controlled RCT with single 6 g gluten bolus.	36	Acute symptoms linked to immune activation.	Median symptom score for nausea increased. IL-2 elevated after 4 hours in 97% of CeD patients and correlated with the severity of nausea.
Goel et al. 2020 (Australia) [55]	Open trial with single 6 g gluten bolus. Non-CeD control group.	25	Quantitative hierarchy of serum cytokines and their relation to symptoms; 187 different cytokines.	12 cytokines detected after challenge, their levels peaking at 4 hours. IL-2 and IL-17A were the earliest to rise and IL-2 had the most prominent elevation. Cytokine levels correlated with symptoms.
Yohannes et al. 2020 (Finland) [56]	Open trial with 10 g of gluten daily for 3 days.	6	Molecular impact of short challenge as measured by RNA sequencing.	Genome-wide transcriptional changes and altered biological pathways in PBMCs.
Leonard et al. 2021 (USA) [57]	Double-blind RCT with either 3 g or 10 g gluten daily for 14 days.	14	Performance of histology, symptoms and various biomarkers to assess CeD activity induced by different gluten doses.	IL-2 and symptoms increased with both doses, IL-2 being the earliest and most sensitive marker. VH:CrD, VCE score, ELISpot, CD4/CD8+ T cells and IELs showed significant changes with 10 g dose only.
Stamnaes et al. 2021 (Norway) [58]	Open trial with 5.7 g gluten daily for 14 days.	19	To find factors explaining variable mucosal response to gluten challenge by investigating global protein expression of intestinal biopsies.	In the comparison of proteome analysis before and after challenge, patients with strong mucosal response to challenge had signs of low-level inflammation already before challenge. The analysis correlated with measured VH:CrD.

APC, antigen-presenting cell; DC, dendritic cell; ELISPot, enzyme-linked immunosorbent spot; EMA, endomysial antibodies; GFD, gluten-free diet; HLA, human leukocyte antigen; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A; IL, interleukin; INF-γ, interferon γ; IP-10, inducible protein 10; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cells; RCT, randomized clinical trial; TGA, transglutaminase 2 antibodies; TNF-α, tumor necrosis factor α; VCE score, Video Capsule Endoscopy score; VH:CrD, Villous height-crypt depth ratio. [1]HLA-DQ2+ (DQA1*05/DQB1*02) individuals aged 18–70 years on a GFD for more than four weeks with an uncertain diagnosis of CeD. [2]Two weeks preceding the challenge there was a safety-face of the trial with a 2-week gluten challenge while using a drug candidate.

Table 3. Gluten challenge studies with duration of >14 days executed in adult celiac disease (CeD) patients.

Study	Study type	Participants	Objectives and outcomes	Main results
Pyle et al. 2005 (USA) [59]	Open trial with 5 g or 10 g of gluten daily for 21 days.	8	Changes in intestinal absorption, serology, and symptoms.	Significant reduction in D-xylose urine excretion and increase in 72-hour fecal fat as signs of malabsorption. None had increase in AGA or TGA. Variable symptom response.
Lähdeaho et al. 2011 (Finland) [60]	Open trial with 1-3 g or 3-5 g of gluten daily for 12 weeks.	25	Gluten dose needed to induce morphological changes in the duodenal mucosa.	Significant decrease in VH:CrD in 67% of participants in both doses. CD3+ IELs increased in 67% of participants, but only with higher dose. 43% seroconverted positive for TGA and 71% had mild to moderate symptoms.
Kelly et al. 2013 (USA) [61]	Double-blind, placebo-controlled RCT with 2.7 g of gluten daily for 6 weeks	43	Gluten-induced changes in intestinal permeability, serology, and symptoms.	No significant change in lactulose-mannitol ratio. Slight increase in gastrointestinal symptoms during first 3 weeks and 30% had positive seroconversion of TGA.
Lähdeaho et al. 2014 (Finland) [62]	Double-blind, placebo-controlled RCT. Part 1: 1.5 g, 3 g or 6 g gluten for 6 weeks; Part 1: 2 g of gluten for 6 weeks.	47/21	1) optimize the dose of gluten used in challenge and 2) assess the gluten-induced changes in histology, symptoms, and quality of life.	Dose-dependent decrease of VH:CrD in part 1. VH:CrD decreased and IEL count increased in part 2. One patient also had positive seroconversion in EMA, whereas there were no significant differences in TGA. Symptoms increased during challenge and returned to baseline by day 70. No change in quality of life.
Lähdeaho et al. 2019 (Finland) [63]	Double-blind, placebo-controlled RCT with 2-4 g gluten daily for 10 weeks.	19	Changes in histology, serology, and symptoms.	Mean decrease of 61% in VH/CrD and increase of 105% in CD3+ IELs, as well as significant increases in TGA, DGPs, and gastrointestinal symptoms.
Taavela et al. 2019 (Finland) [64]	Open trial with 4 g gluten daily for 10 weeks. Non-CeD control group.	15	Changes in histology, immunohistochemical markers, and RNA markers using PAXgene-fixated duodenal biopsies.	Digital VH:CrD revealed mucosal deterioration in all participants compared to 80% with Marsh-Oberhuber classification. Significant increase in CD3+, γδ+, and CD138+ IELs. Molecular morphometry showed similar changes from baseline to post-challenge as histomorphometric measurements.

Dotsenko et al. 2021 (Finland) [65]	Open trial with 2-4 g of gluten daily for 10 weeks. Non-CeD control group.	15	Early transcriptomic changes with RNA sequencing and their correlation with histology.	Genome-wide transcriptional changes and significant decrease in VH:CrD and increase in CD3+ IELs. There was a correlation between the changes in gene expression and histology.
Schuppan et al. 2021 (Finland) [66]	Double-blind, placebo-controlled RCT with 3 g gluten daily for 6 weeks.	40	Changes in histology, serology, and symptoms.	VH:CrD decreased and IEL count increased significantly from baseline and 16% seroconverted positive for TGA. Gastrointestinal symptoms increased but returned to baseline at follow-up visit.
Murray et al. 2022 (USA) [67]	Double-blind, placebo-controlled RCT with 2 g of gluten daily for 6 weeks.	22	Changes in histology, serology, urine GIP, and symptoms.	Significant decrease in VH:CrD and increase in IELs and abdominal pain, bloating and tiredness. No significant change in TGA or DGPs.

AGA, anti-gliadin antibodies; DGP, antibodies to deamidated gliadin peptide; EMA, endomysium antibodies; GIP, gluten immunogenic peptides; IEL, intraepithelial lymphocyte; RCT, randomized clinical trial; TGA, transglutaminase antibodies; VH:CrD, villous heigh-crypt depth ratio