DR JASON TYE-DIN (Orcid ID : 0000-0001-7687-9654)

DR KENNETH E TRUITT (Orcid ID : 0000-0001-9350-0212)

DR ROBERT PAUL ANDERSON (Orcid ID : 0000-0002-0764-7267)

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MASKED BOLUS GLUTEN CHALLENGE LOW IN FODMAPS IMPLICATES NAUSEA AND VOMITING AS KEY SYMPTOMS ASSOCIATED WITH IMMUNE ACTIVATION IN TREATED COELIAC DISEASE

Short Title: Symptoms and reactivation of gluten immunity

Authors: Daveson, A James M.^{†1}; Tye-Din, Jason A.^{†2,3,4,5}; Goel, Gautam⁶; Goldstein, Kaela E.⁶; Hand, Holly L.⁶; Neff, Kristin M.⁶; Williams, Leslie J.⁶; Truitt, Kenneth E.⁶; Anderson, Robert P.^{6††} on behalf of the RESET CeD Study Group‡

Affiliations:

¹Faculty of Medicine, University of Queensland, Brisbane, Queensland, Australia

²Immunology Division, The Walter and Eliza Hall Institute, Parkville, VIC, Australia

³Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia

⁴Department of Gastroenterology, The Royal Melbourne Hospital, Parkville, VIC, Australia

⁵Centre for Food & Allergy Research, Murdoch Children's Research Institute, Parkville, VIC, Australia

⁶ImmusanT, Inc., Cambridge, Massachusetts, USA

† These authors contributed equally to this work.

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†† To whom correspondence should be addressed: Dr. Robert P. Anderson, ImmusanT Inc., One Kendall Square, Suite 7201, Cambridge, MA 02139, or at banderson99133@gmail.com

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‡ A. Adams (AB Clinical Trials, Las Vegas, NV, USA), J. Andrews (Royal Adelaide Hospital, Adelaide, SA, AUS), C. Behrend (Grand Teton Research Group, Idaho Falls, ID, USA), G. Brown, (Alfred Hospital, Melbourne, VC, AUS), S. Chen Yi Mei (Eastern Health-Box Hill Hospital, Box Hill, VC, AUS), A. Coates (Gastroenterology Associates of Western Michigan, PLC d.b.a. West Michigan Clinical Research Center, Wyoming, MI, USA), A.J.M Daveson (Coral Sea Clinical Research Institute, North Mackay, QLD, AUS, and Wesley Medical Research - The Wesley Hospital, Auchenflower, QLD, AUS), A. DiMarino (Thomas Jefferson University Hospital, Philadelphia, PA, USA), H. Ee (Department of Gastroenterology, Sir Charles Gairdner Hospital, Nedlands, WA, AUS), D. Elliott (University Of Iowa, Iowa City, IA, USA), R. Epstein (ActivMed Practices & Research, Inc., Portsmouth, NH, USA), B. Feyen, (PMG Research of McFarland Clinic, Ames, IA, USA), R. Fogel (Clinical Research Institute of Michigan, Chesterfield, MI, USA), K. Friedenberg (Great Lakes Gastroenterology Research, Mentor, OH, USA), R. Gearry (Department of Medicine, University of Otago, Christchurch, NZ), M. Gerdis (Drug Trials America, Hartsdale, NY, USA), M. Goldstein (Long Island Gastrointestinal Research Group, Great Neck, NY, USA), V. Gupta (Alliance Medical Research LLC, Lighthouse Point, FL, USA), R. Holmes (PMG Research of Winston-Salem, LLC,

Winston-Salem, NC, USA), G. Holtmann (The University of Queensland - Princess Alexandra Hospital, Woolloongabba, QLD, AUS), S. Idarraga (Allegiance Research Specialists, Wauwatosa, WI, USA), G. James (Digestive Health Research, LLC, Hermitage, TN, USA), T. King, (Auckland Clinical Studies Limited, Auckland, NZ), T. Klein (Heartland Research Associates, LLC, Wichita, KS, USA), S. Kupfer (The University of Chicago Medical Center, Chicago, IL, USA), B. Lebowitz (Celiac Disease Center at Columbia University, New York City, NY, USA), J. Lowe (Advanced Research Institute, Ogden, UT, USA), J. Murray (Mayo Clinic, Rochester, MN, USA), E. Newton (Omega Medical Research, Warwick, RI, USA), D. Quinn (P3 Research Limited, Wellington, NZ), D. Radin (Stamford Therapeutics Consortium, Stamford, CT, USA), T. Ritter (Texas Digestive Disease Consultants, Southlake, TX, USA), H. Stacey (Diablo Clinical Research, Inc., Walnut Creek, CA, USA), C. Strout (Coastal Carolina Research Center, Mount Pleasant, SC, USA), R. Stubbs (P3 Research Limited, Havelock North, NZ), S. Thackwray (Clinical Trials Centre - University of the Sunshine Coast, Sippy Downs, QLD, AUS), V. Trivedi (PMG Research, Inc., d/b/a PMG Research of Piedmont Healthcare, Statesville, NC, USA), J. Tye-Din (The Royal Melbourne Hospital - The Walter And Eliza Hall Institute of Medical Research, Parkville, VIC, AUS), J. Weber (Center for Digestive Health, Troy, MI, USA), S. Wilson (Ocean State Clinical Research Partners, Lincoln, RI, USA)

ABSTRACT

Background: FODMAPs in gluten-containing foods, and participant anticipation of a harmful (“nocebo”) effect may contribute to acute symptoms after gluten challenge.

Aim: To establish acute gluten-specific symptoms linked to immune activation in coeliac disease.

Methods: 36 coeliac disease patients on a gluten-free diet receiving placebo in the RESET CeD trial were included. Double-blind, bolus vital wheat gluten (~6-gram gluten protein) and sham challenges low in FODMAPs were consumed 2-weeks apart.

Assessments included daily Coeliac Disease Patient Reported Outcome (CeD PRO) symptom scores (0-10), adverse events, and serum interleukin-2 (baseline and 4 h)

Results: Median CeD PRO score for nausea increased most (sham: 0 versus gluten: 5.5; $p<0.001$). Apart from tiredness (1 versus 4, $p=0.005$), and headache (0 versus 2,

p=0.002), changes in other symptoms were small or absent. Only nausea increased significantly in occurrence with gluten (11% versus 69%, p<0.001). Without nausea occurring, only tiredness and flatulence were common after gluten. Nausea (6% versus 61%, p<0.001; median onset: 1:34h) and vomiting (0% versus 44%, p<0.001; 1:51h) were the only adverse events more common with gluten than sham. Interleukin-2 was always below the level of quantitation (0.5 pg/ml) at baseline, and after sham. Interleukin-2 was elevated after gluten in 97% of patients (median fold-change: 20), and correlated with severity of nausea ($r_s=0.49$, p=0.0025) and occurrence of vomiting (p=0.0005).

Conclusions: Nausea and vomiting are implicated as relatively specific indicators of acute gluten ingestion, and correlate with immune activation. IBS-like symptoms without nausea are unlikely to indicate recent gluten exposure.

Key words: Coeliac disease, cytokine release, diagnostic, gluten, gluten-free diet, interleukin-2, patient reported outcome.

Abbreviations: fermentable oligo-, di-, monosaccharides, and polyols (FODMAPs), Human leukocyte antigen-DQ (HLA-DQ), irritable bowel syndrome (IBS), parts per million (ppm).

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INTRODUCTION

Many patients with coeliac disease fail to achieve intestinal mucosal recovery despite reporting strict avoidance of dietary gluten (1, 2). This is clinically important as persistent intestinal mucosal injury carries increased risks of many complications such as osteoporosis, normally associated with untreated coeliac disease (3, 4). Persistent intestinal mucosal injury appears to be explained by recurrent, inadvertent gluten exposure (5). Indeed, patients and clinicians may not be able to accurately recognise episodes of gluten ingestion if reported symptoms are attributed to irritable bowel syndrome (IBS) (6, 7). The symptoms of IBS may be triggered by poorly absorbed,

fermentable, short-chain carbohydrates (FODMAPs) present in many gluten-free foods as well as being enriched in wheat flour, the commonest source of gluten in Western diets (8, 9). Understanding the timing and nature of acute, immune-mediated symptoms caused by gluten in patients with coeliac disease following a gluten-free diet could allow patients and health care professionals to better recognise when gluten has been ingested, and improve avoidance strategies. Additionally, regulatory authorities have emphasised the importance of patient reported outcomes as efficacy endpoints when testing novel therapeutics for coeliac disease (10).

Although the immune mechanisms are debated and supportive blood biomarkers are lacking, onset of digestive symptoms is about two hours after food exposure in non-IgE immune-mediated food hypersensitivities (11). In contrast, clinical descriptions of coeliac disease, a T cell-mediated condition, emphasise chronic symptoms similar to IBS (6, 7). There are, however, reports of acute reactions to gluten in patients with coeliac disease on gluten-free diet (6, 7), but until recently there appeared to be no mechanistic explanation, and well-controlled gluten food challenge studies addressing this issue are absent.

Recently, we reported systemic cytokine release in conjunction with acute digestive symptoms in patients with coeliac disease two hours after injection of gluten peptides that directly activate gluten-specific CD4+ T cells (12). Subsequently, we showed that patients with coeliac disease on gluten-free diets also experience a cytokine release phenomenon after consuming gluten (12). Pronounced elevation of interleukin-2, a cytokine released by antigen-activated T cells, dominates the changes in serum cytokines after gluten ingestion (13). Detailed time-course studies indicate peak serum levels of interleukin-2 occur four hours after gluten challenge (12), and correlate with overall severity of digestive symptoms, and nausea in particular (14).

Previously, at least three important factors have obscured understanding acute symptoms caused by gluten in coeliac disease: (1) there has been no biomarker to quantify acute gluten-mediated immune activation, (2) the potential for a nocebo effect, defined as participant anticipation of a harmful response (15), and (3) without matching the active

and control food challenges for low FODMAPs content, there is the possibility that active and/or sham may cause IBS-like symptoms unrelated to immune-mediated effects of gluten (16, 17). To date there have been no studies that have addressed each of these issues.

The aim of the present study was to establish acute gluten-specific symptoms linked to immune activation in coeliac disease.

METHODS

Study Design

Participants were enrolled in the “RESET CeD” study that tested the efficacy of an investigational immunotherapy (Nexvax2) in coeliac disease patients established on a gluten-free diet (ClinicalTrials.gov identifier: NCT03644069). The study design is shown in Figure 1. The present report addresses two exploratory objectives of the RESET CeD study to assess the tolerability of gluten food challenge, and evaluate the relationship between elevations in serum cytokines and symptoms after gluten challenge. We compared a self-reported daily recall of coeliac disease-associated symptoms for the day of sham and gluten food challenges using the “Celiac Disease Patient Reported Outcome” (CeD PRO[®]) instrument (18). CeD PRO data were corroborated using adverse events recorded by study site staff, and compared to elevations of serum interleukin-2 from baseline to four hours after food challenge. The symptoms patients had expected would be worst after gluten were compared to the actual symptoms rated most severe after the gluten challenge. The study was done in accordance with the International Conference on Harmonisation for Good Clinical Practice. ICON plc (Dublin 18, Ireland) managed the study. There were 26 study sites in the United States, four in New Zealand & nine in Australia that enrolled volunteers (Table S1). Local ethics committees (Table S2) approved the study.

Participants and clinical interventions

All patients gave written, informed consent prior to undergoing any trial-related procedures. Eligibility criteria for screening and randomisation are listed in Table S3.

Participants were required to be aged between 18 to 70 years, produce documentary evidence of duodenal villous atrophy and elevated coeliac disease-specific serology when coeliac disease was diagnosed, and to have followed a gluten-free diet for at least one year. Requirements for patients to be randomised to treatment included being positive for HLA-DQ2.5 and that they showed deterioration in digestive symptoms within six hours after the unmasked screening gluten challenge (measured by the Global Symptom Survey described by Tye-Din et al) (14), or experienced a gastrointestinal adverse event of at least moderate severity within 24 hours. For the present study, data were analysed only for those patients randomised to placebo treatment who completed both a masked sham and a masked active gluten food challenge, and had interleukin-2 and patient reported outcome assessments recorded for the days of both masked food challenges. Not all placebo-treated participants were included in the present study because sites were alerted to discontinue dosing on 25 June 2019 after findings of an interim analysis indicated the primary endpoint of the RESET CeD study would not be achieved (Figure S1).

Assessments

Data collected during screening included demographics, medical history, a review of prior/concomitant medications and procedures, a “Celiac disease characteristics survey” (14), serology (QUANTA Lite® R h-tTG IgA and Gliadin IgA II, INOVA Diagnostics, San Diego, California, USA; performed by ARUP, Salt Lake City, UT), and HLA-DQ genotype (UCLA Immunogenetics Center, Los Angeles, CA). Adverse events were recorded throughout the study. MedDRA Preferred Terms (v21.0) were used to code adverse events, and their severity was graded according to Common Terminology Criteria for Adverse Events, Version 4.03. Throughout the treatment period including when masked food challenges were administered, patients recorded their responses to the seven digestive symptoms, headache and tiredness items in the CeD PRO (a whole number from 0 for no symptoms, to 10 for worst possible). Patients provided daily CeD PRO responses between 6:00 pm and midnight each evening using a hand-held electronic patient-reported outcome device. Serum was separated from blood collected at baseline and at four hours after masked gluten food challenges. As described by Tye-Din et al (14), Covance Laboratories, Inc. (Immunochemistry Services, Chantilly, Virginia USA)

measured serum interleukin-2 levels using a validated V-PLEX electrochemiluminescence assay (Meso Scale Discovery; Rockville, Maryland, USA). Based on the unmasked gluten challenge reported by Tye-Din et al (14), Covance Laboratories established that the lower level of quantitation for serum interleukin-2 was 0.5 pg/ml. Serum interleukin-2 levels above this level after food challenge were considered elevated when the baseline was reported <0.5 pg/ml.

Masked Food challenges

Rutgers Food Innovation Center (450 Broad Street, Bridgeton, N.J.) developed and manufactured food challenge pouches that were matched in appearance and contained either 11 g vital wheat gluten flour (Manildra Milling Corporation; Nowra NSW, Australia), or 11 g of a blend of rice starch, guar gum, and potato protein (8.75:1.25:1 by weight) (rice starch: Ingredion Inc., Indianapolis, IN, USA; guar gum: TIC Gums, White Marsh, MD, USA; and potato protein: Avebe, Veendam, The Netherlands). The content of the active challenge was the same as that described by Tye-Din et al (14). Before the study commenced, food challenge material was analysed for microbes, protein and water (Eurofins Microbiology Laboratories Inc., Lancaster, Pennsylvania, USA), gluten immunoreactivity (Neogen Corp., Lansing, Michigan, United States), and FODMAPs (Monash University, Melbourne, Victoria, Australia). Food composition analyses are summarised in Table S4. The gluten and sham food challenges and flavoring were low in FODMAPs according to (19), gluten levels were <20 parts per million (ppm) for the sham and flavouring, and over a 15-month storage period at room temperature the contents of food challenge pouches remained free of enteropathogens. Patients consumed the contents of one pouch of gluten or sham and flavouring (Watermelon Aqua Fresca Blend Non-GMO 3 g; True Citrus Co.; Baltimore, MD, USA) mixed to a smooth consistency in 100 ml bottled water over no more than two minutes. Patients then consumed 150 ml bottled water. Site staff and patients were unaware of the contents of the food challenge pouch. Patients remained at the study site for at least four hours after these food challenges.

Statistical analyses

No formal power calculation was undertaken for exploratory objectives related to masked food challenges in the RESET CeD Study. Non-parametric tests were used to assess the significance of differences in paired (Wilcoxon signed rank test) or unpaired (Mann-Whitney test) observations, and to assess correlations (Spearman's coefficient). All statistical tests were 2-tailed. P-values were adjusted for false discovery rate caused by multiple comparisons using the Benjamini-Hochberg method. Adjusted P-values were considered significant when less than or equal to 0.05. Summary and significance statistics were computed using a combination of Graphpad Prism V7.0d and Mathworks Matlab Ver 9.5 (R2018b). All figures were plotted using Graphpad Prism V7.0d.

RESULTS

Patient characteristics

Disposition of patients enrolled in the RESET CeD study is shown in Figure S1. Altogether, 383 volunteers were screened. Among the 310 eligible recruits who completed the unmasked gluten challenge at screening, 64 were excluded from randomisation because they had minimal or no deterioration in gastrointestinal symptoms by six hours after consuming gluten. Out of the remaining 246 patients, 179 were randomised to receive investigational product with 86 receiving placebo. Overall, 36 placebo-treated patients completed paired sham and gluten masked food challenges and were included in the analysis. For the 36 patients included in the analysis, masked food challenges were performed over the period from 12 December 2018 to 24 May 2019. Twenty-one (58%) were from the United States, 3 (8%) were from New Zealand, and 12 (33%) were from Australia. Participants had a median age of 42 years (interquartile range: 34-54), 25 (69%) were women, and the median period since diagnosis of coeliac disease was five years (interquartile range: 2-8). All patients were positive for HLA-DQ2.5, and six (17%) were homozygotes. Three (8%) patients had levels of transglutaminase 2 IgA that were equivocal or positive, and another four (11%) had levels of deamidated gliadin peptide IgG that were equivocal or positive at screening.

Patient reported outcomes after masked food challenges

Each evening, patients rated the severity of the nine symptoms in the CeD PRO from 0 (“no symptoms”) to 10 (“worst possible”). Over the three days immediately before and after sham and gluten challenges median daily scores for “gas” and “tiredness” were between 0 and 2, but for the other seven symptoms rated by patients in the CeD PRO their median scores were consistently 0 (data not shown). CeD PRO symptom scores on the day of sham food challenge were not significantly different from the day before, whereas all nine CeD PRO item scores on the day of gluten food challenge were significantly higher than the day before (Table S5). Symptom scores in the CeD PRO for the day of sham and gluten challenges are shown in Figure 2 and summarized in Table 1. Overall, nausea was the most severe symptom on the day of gluten challenge (median: 5.5, interquartile range: 0 - 9) compared to sham (0, 0 – 0). The median net increase in nausea score for individual patients comparing the day of sham to the day of gluten challenge was +4.5 ($p = 3 \times 10^{-7}$ by Mann-Whitney U Test adjusted for false discovery rate by Benjamini-Hochberg method). Nausea was also the symptom that had the greatest increase in occurrence (score of 1 or greater) between sham and gluten challenges (11% versus 69%, $p = 2 \times 10^{-7}$ by Fisher Exact Test adjusted for false discovery rate by Benjamini-Hochberg method) (Table 1). Although there were trends for the occurrence of all symptoms except gas to be higher with gluten challenge, none were statistically significant. Gluten challenge was, however, associated with significant increases in the severity of tiredness, headache, abdominal pain, abdominal cramping, diarrhoea and loose stool, but their median severity on the day of gluten challenge (range: 0 – 1.5) was modest compared to nausea (5.5).

CeD PRO scores for nausea on the day of gluten challenge had a bimodal distribution (Figure 2A), and occurrence of nausea appeared to be linked to other symptoms after gluten challenge in the heat-map profiling individual patient’s symptoms (Figure S2). To test whether nausea might be a sentinel for a symptom complex triggered by gluten exposure in patients with coeliac disease, CeD PRO data were compared between patients who did experience nausea after gluten ($n = 25$) and those who did not ($n = 11$). Tiredness, headache, abdominal pain, abdominal cramping, diarrhea, and loose stool were each significantly more severe and more common in patients who experienced

nausea (Table 2). At least 60% of patients who experienced nausea also reported tiredness, headache, abdominal pain, abdominal cramping, diarrhea, and/or loose stool after gluten, whereas only tiredness and gas were common symptoms in patients who did not report nausea after gluten challenge.

Collectively, patient reported outcomes on the days of masked food challenges indicated gluten challenge either induced a symptom complex dominated by nausea, or otherwise symptoms were mild or absent and indistinguishable from those after sham food challenge. Patients with coeliac disease on gluten-free diet did not report IBS-like symptoms after they consumed gluten unless they also experienced nausea.

Adverse events after masked food challenges

Adverse events with onset during the 24 hours after sham and gluten food challenges are summarized in Table 3, and shown as a heat-map for individual patients in Figure S2. The adverse events that were significantly more common after gluten than sham food challenge were nausea ($p = 1 \times 10^{-5}$ by Mann-Whitney U Test adjusted for false discovery rate by Benjamini-Hochberg method) and vomiting ($p = 3 \times 10^{-5}$). Nausea was recorded for 22 (61%) patients and vomiting for 16 (44%) patients after gluten challenge, which compared to 2 (5%) patients who had nausea and none who vomited after sham food challenge. Trends for more frequent diarrhoea, fatigue, abdominal pain, bloating, and headache after gluten compared to sham did not reach statistical significance. The onset of nausea was typically one and half-hours after consuming gluten (median: 1:34 h, interquartile range: 1:26 - 1:46 h) and if it occurred, vomiting followed about half-an-hour later (median onset after gluten: 1:51 h, interquartile range: 1:24 – 2:17 h).

Interleukin-2 elevations and symptoms after masked food challenges

Serum interleukin-2 was always below the level of quantitation (0.5 pg/ml) before and four hours after sham challenge, and also before gluten challenge. In contrast, 35 (97%) patients had serum interleukin-2 levels above 0.5 pg/ml four hours after gluten challenge (median: 10, interquartile range: 3 – 43 pg/ml; fold-change from baseline: 20, 6 – 84), which was significantly different from sham challenge ($p < 0.0001$, Wilcoxon Test).

Serum interleukin-2 at four hours after gluten was significantly higher in patients with CeD PRO nausea scores of 1 or more ($n = 25$, median: 21, interquartile range: 6 – 47 pg/ml) than in those with a nausea score of 0 ($n = 11$, median 2, interquartile range: 2 – 10; $p = 0.0012$ by Mann Whitney test). There were significant correlations between serum interleukin-2 levels at four hours after gluten and severity of nausea assessed by CeD PRO (Spearman $r = 0.54$, $p = 0.0008$) and severity of nausea as an adverse event (Spearman $r = 0.49$, $p = 0.0025$) (Figure 3). Serum interleukin-2 at four hours was also significantly higher in patients who vomited (median: 44, interquartile range: 9 – 90 pg/ml) than in patients who did not vomit (median 6, interquartile range: 2 – 11; $p = 0.0005$ by Mann Whitney test). Other symptoms assessed in the CeD PRO were not significantly correlated with serum interleukin-2 at four hours after gluten.

Interestingly, serum interleukin-2 levels at four hours after gluten were elevated in all five asymptomatic patients who recorded no symptom scores above 0 and had no adverse events after gluten (median: 1.6, interquartile range: 0.7 – 4.7). The single patient with interleukin-2 levels less than 0.5 pg/ml before and four hours after gluten was distinguished as the only patient who had severity scores between 3 and 8 for all CeD PRO items on the day of both the gluten as well as sham food challenge (Figure S2).

Actual and expected symptoms after gluten

Vomiting occurred, or nausea was scored the most severe of any symptom after gluten challenge in 22 (61%) patients (Table S6). For the other patients, tiredness was the most severe symptom for five (14%), gas in three (8%), diarrhoea in one (3%), and five (14%) patients reported no symptoms. In contrast, at the start of the RESET CeD Study during screening, patients stated what they expected would be their worst symptom after gluten challenge. Twelve patients recorded diarrhoea (33%), ten (28%) abdominal pain, four (11%) nausea, two (6%) each for vomiting, headache, gas, and bloating, and one (3%) for fatigue, and one (3%) did not know. Only ten (28%) patients correctly anticipated their worst symptom after the masked gluten challenge administered in this study.

DISCUSSION

Acute gluten-associated symptoms in coeliac disease were evaluated in patients on a gluten-free diet using a double-blind, sham-controlled food challenge low in FODMAPs, and correlated with serum interleukin-2 elevation as a relevant biomarker of immune activation. The bolus sham food challenge was not associated with significant symptom induction, but the gluten challenge was associated with significant induction of digestive symptoms, as well as headache and tiredness. Nausea was clearly the symptom that differed most in severity and occurrence on the day of gluten challenge compared to sham challenge, and was the only one of nine items assessed by a patient reported outcome instrument that showed significant correlation between severity and serum levels of interleukin-2 at four hours. After gluten, patients either experienced digestive symptoms dominated by nausea and sometimes vomiting, or otherwise symptoms were mild or absent and indistinguishable from those after sham food challenge. During the day of this gluten food challenge low in FODMAPs, patients with coeliac disease on gluten-free diet did not report IBS-like symptoms after they consumed gluten unless they also experienced nausea. Overall, elevation in serum interleukin-2 was 97% sensitive and 100% specific for patients exposed to gluten compared to sham, whereas the symptom complex with nausea as its sentinel symptom was 69% sensitive and 89% specific for patients exposed to gluten compared to sham.

Although the study design was novel and addressed several important factors limiting interpretation of previous studies in this field, there were several limitations. The composition and amount of gluten in the active gluten challenge was the same as that in an unmasked challenge recently reported by Tye-Din et al (14), which was tolerated but clearly caused a robust symptomatic and immunological response. However, the amount of gluten in the food challenge was restricted to a single dose level, estimated to be six grams of gluten protein (about half the amount consumed daily in an unrestricted diet), and only a single challenge format was tested. FODMAPs content in the food challenge was deliberately chosen to be low to minimise the confounding effect of non-gluten dietary constituents. Future studies could consider addressing the effects of food challenge format, gluten dose, and whether to include additional matched food challenges

that have FODMAPs without gluten or in conjunction with gluten. Our study required patients be positive for HLA-DQ2.5 (representing > 90% of patients with coeliac disease), following a gluten-free diet for at least one year, and be at least moderately symptomatic after an unmasked food challenge four months earlier. Therefore, less restrictive eligibility criteria may have resulted in patients experiencing less prominent digestive symptoms, or perhaps symptoms in patients negative for HLA-DQ2.5 that were qualitatively different. Only interleukin-2 was assessed, but we now know that more modest elevations of several other cytokines follow gluten exposure. Future studies could consider measuring these other cytokines (CCL20, IL-6, CXCL9, CXCL8, IFN- γ , IL-10, IL-22, IL-17A, TNF- α , CCL2, and amphiregulin) (13).

Designing and confirming the sham and active gluten challenges to be low in FODMAPs may account for the low symptom response to the sham, and also explain why IBS-like symptoms were not as prominent as patients expected after gluten challenge.

Administering six grams of gluten protein in standard bread would have resulted in high exposure to FODMAPs (8), whereas the amount of vital wheat gluten flour ingested in our challenge resulted in low exposure to FODMAPs. We suggest that this aspect of the study design, and gluten-reactive CD4⁺ T cells being located in the mucosa of the proximal small intestine (20), is likely to explain the prominence of acute symptoms associated with the upper gastrointestinal tract after gluten challenge. “Real-life” exposures to gluten in patients with coeliac disease on gluten-free diet most likely result from ingestion of whole food that contains both gluten and FODMAPs. Future studies could address the types of symptoms prominent after challenge with gluten-free FODMAPs in patients with coeliac disease. Our findings suggest nausea is the symptom most indicative of recent gluten exposure, but given the broad gastrointestinal symptom responses triggered by gluten-free FODMAP challenge in patients with irritable bowel syndrome (16, 17), nausea may not be as specific for gluten when FODMAPs are consumed.

IBS-associated symptoms are a common complaint in patients with coeliac disease, especially in those less adherent to gluten-free diet (21). The findings of our study are

431 limited to the day of a substantial gluten exposure when nausea predominates, and are
432 informative of acute symptoms within hours of gluten ingestion. Duodenal injury related
433 to chronic gluten exposure is found in many patients with coeliac disease on a gluten-free
434 diet (1, 2). Therefore, IBS-like symptoms being common in coeliac disease may be a
435 non-specific effect of mucosal inflammation rather than directly linked to gluten-
436 mediated immune activation (22). This is an important clinical consideration, as both
437 patients with coeliac disease and their treating clinicians frequently attribute persistent
438 symptomatology to accidental gluten exposure. Our findings suggest that it is important
439 to consider non-gluten driven causes for symptomatology such as FODMAPs driving
440 IBS. Furthermore, as symptom-based endpoints for clinical trials in coeliac disease are
441 likely to be significantly confounded by the effects of FODMAPs, it will be important to
442 distinguish between IBS-like symptoms in chronic disease related to gluten and/or
443 FODMAPs exposure, and nausea-predominant acute symptoms linked to cytokine release
444 within hours after gluten exposure.

445
446 Findings in the current study build upon our previous reports suggesting that objective
447 biomarkers such as serum interleukin-2 may ultimately serve to augment or eventually
448 replace subjective symptom assessments as an acceptable efficacy endpoint in
449 therapeutics trials for coeliac disease. The mechanistic link between systemic cytokine
450 release and digestive symptoms is a focus of active research. At present, the correlation
451 between serum interleukin-2 levels and symptoms after gluten challenge should be
452 considered an association rather than a causal relationship. Immune activation and
453 cytokine levels in the duodenal mucosa may prove to be more strongly correlated with
454 symptoms than serum cytokines following gluten challenge in coeliac disease.

455
456 Dietary education is the cornerstone of management in patients newly diagnosed with
457 coeliac disease (9). Understanding what symptoms are indicative of recent gluten
458 exposure could allow patients to recognise and avoid foods or situations that increase the
459 likelihood of gluten contamination and clinicians to advise accordingly. In the present
460 study, patients were generally unable to anticipate the symptoms that were experienced
461 after gluten. Our findings suggest that symptoms associated with IBS are incorrectly

attributed to recent exposure to gluten, even though IBS is common in patients treated for coeliac disease (21). Our findings also highlight the limitations of symptoms in general as a clear-cut readout of gluten ingestion and, for clinicians, underscore the value of employing sensitive objective measures of gluten exposure, such as gluten immunogenic peptide assays of urine (23) or faeces (24, 25), to correlate symptoms with true episodes of gluten exposure.

Collectively these findings implicate nausea, sometimes with vomiting, linked to reactivation of gluten immunity as the sentinel symptom of acute gluten exposure in patients following a gluten-free diet. Elevation in serum interleukin-2 was more responsive than symptom assessments after gluten challenge, and may provide a sensitive and objective biomarker to support endpoint assessments in therapeutic trials.

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Figure legends

Figure 1. Overview of the RESET CeD study in HLA-DQ2.5+ patients with treated coeliac disease. After screening, eligible patients were randomised 1:1 to receive placebo or active investigational product (Nexvax2). The placebo treatment consisted of subcutaneous injections of 0.9% sodium chloride two-times weekly. Sham and gluten masked food challenges were completed during week 12 and 14 of the treatment period, respectively. Data from placebo-treated patients were used to determine the effects of gluten on acute symptoms, and their relation to systemic cytokine release. Daily patient reported outcomes, adverse events and elevations of serum interleukin-2 at four hours after food challenge were analysed. The active food challenge in week 14 was vital wheat gluten and contained 6 g gluten protein. The sham and gluten challenges were both low in FODMAPs and taken as a single bolus mixed in water after fasting overnight.

Figure 2. Daily symptom scores recorded by patients using the CeD PRO instrument for the day of masked food challenges. Median and interquartile ranges are shown.

Figure 3. Nausea and vomiting compared to serum interleukin-2 at four hours after gluten challenge. **A** Severity of nausea recorded as an adverse event with patients who vomited indicated by circles. **B** Severity of self-reported nausea measured by CeD PRO with patients who vomited indicated by circles. Spearman's rank-order correlation, and P-value is shown.

Table 1. Symptoms on the day of sham and gluten challenges

	CeD PRO scores (0 – 10)				Symptom occurrence			
	Median (interquartile range)				Number (%) of patients out of 36 with item score ≥ 1			
	Sham	Gluten	Difference [†]	P-value [‡]	Sham	Gluten	Difference [†]	P-value ^{‡,§}
Nausea	0 (0 - 0)	5.5 (0 - 9)	4.5 (0 - 8)	<0.001	4 (11)	25 (69)	21 (58)	<0.001
Tiredness	1 (0 - 3)	4 (1 - 7)	1.5 (0 - 4)	0.005	20 (56)	29 (81)	9 (25)	0.063
Headache	0 (0 - 1)	2 (0 - 5)	1 (0 - 4)	0.002	10 (28)	21 (58)	11 (31)	0.075
Pain	0 (0 - 1)	1 (0 - 3)	0 (0 - 2)	0.015	10 (28)	20 (56)	10 (28)	0.055
Cramping	0 (0 - 1)	1 (0 - 4)	0 (0 - 2)	0.017	9 (25)	19 (53)	10 (28)	0.065
Diarrhea	0 (0 - 0)	0 (0 - 5)	0 (0 - 2)	0.021	7 (19)	17 (47)	10 (28)	0.070
Loose Stool	0 (0 - 1)	0.5 (0 - 5)	0 (0 - 1)	0.032	9 (25)	18 (50)	9 (25)	0.065
Bloating	0 (0 - 2)	0.5 (0 - 3)	0 (0 - 2)	0.292	15 (42)	18 (50)	3 (8)	0.716
Gas	1 (0 - 1)	1 (0 - 3)	0 (-1 - 1)	0.857	24 (67)	23 (64)	-1 (-3)	1

[†] “Difference” is an individual’s symptom score after gluten challenge minus that after sham challenge. [‡] CeD PRO symptom scores compared by Mann-Whitney U Test, and [§] occurrence of symptoms compared by Fisher’s exact test, both adjusted for false discovery rate by Benjamini-Hochberg method

Table 2. Symptoms in patients with or without nausea on the day of gluten challenge

	CeD PRO scores (0 – 10)			Symptom occurrence		
	Median (interquartile range)			Number (%) of patients with item score ≥ 1		
	Nausea (n = 25)	No nausea (n = 11)	P-value [†]	Nausea (n = 25)	No nausea (n = 11)	P-value [‡]
Tiredness	4 (3 - 8)	1 (0 - 5)	0.009	23 (92)	6 (54)	0.024
Headache	3 (0.5 - 6)	0 (0 - 0)	0.008	19 (76)	2 (18)	0.010
Pain	2 (1 - 4.5)	0 (0 - 0)	<0.001	20 (80)	0 (0)	<0.001
Cramping	2 (0 - 4)	0 (0 - 0)	0.009	17 (68)	2 (18)	0.016
Diarrhea	2 (0 - 6)	0 (0 - 0)	0.005	16 (64)	1 (9)	0.007
Loose Stool	1 (0 - 7.5)	0 (0 - 0)	0.005	17 (68)	1 (9)	0.007
Bloating	1 (0 - 3)	0 (0 - 2)	0.074	15 (60)	3 (27)	0.167
Gas	1 (0 - 3)	0 (0 - 3)	0.254	18 (72)	5 (45)	0.153

[†] CeD PRO symptom scores compared by Mann-Whitney U Test, and [‡] occurrence of symptoms compared by Fisher’s exact test, both adjusted for false discovery rate by Benjamini-Hochberg method.

Table 3. Adverse events with onset the day of food challenge

Adverse event	Sham	Gluten	Sham vs gluten
	Number (%) of patients	Number (%) of patients	P-value [†]
Nausea	2 (6)	22 (61)	<0.001
Vomiting	0	16 (44)	<0.001

Diarrhoea	3 (8)	10 (28)	0.237
Fatigue	2 (6)	8 (22)	0.254
Abdominal pain	3 (8)	7 (19)	0.658
Bloating	0	6 (17)	0.125
Headache	0	4 (11)	0.286
Burping	1 (3)	3 (8)	0.921
Gastro-oesophageal reflux	2 (6)	1 (3)	1
"Brain fog"	0	2 (6)	0.822
Chills/Flu-like	0	2 (6)	0.822
Rash	0	1 (3)	1
Other	2 (6)	3 (8)	1

† CeD PRO symptom scores compared by Mann-Whitney U Test, and adjusted for false discovery rate by Benjamini-Hochberg method.

Figure 1. Over view of the ReSet CeD study in HLA-DQ2.5+ patients with treated coeliac disease. After screening, eligible patients were randomised 1:1 to receive placebo or active investigational product (Nexvax2). The placebo treatment consisted of subcutaneous injections of 0.9% sodium chloride two-times weekly. Sham and gluten masked food challenges were completed during week 12 and 14 of the treatment period, respectively. Data from placebo-treated patients were used to determine the effects of gluten on acute symptoms, and their relation to systemic cytokine release. Daily patient reported outcomes, adverse events and elevations of serum interleukin-2 at four hours were analysed. The active food challenge in week 14 was vital wheat gluten and contained 6 g gluten protein. The sham and gluten challenges were both low in FODMAPs and taken as a single bolus mixed in water after fasting overnight.

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Author/s:

Daveson, AJM;Tye-Din, JA;Goel, G;Goldstein, KE;Hand, HL;Neff, KM;Williams, LJ;Truitt, KE;Anderson, RP;Adams, A;Andrews, J;Behrend, C;Brown, G;Mei, SCY;Coates, A;Daveson, AJM;DiMarino, A;Ee, H;Elliott, D;Epstein, R;Feyen, B;Fogel, R;Friedenberg, K;Gearry, R;Gerdis, M;Goldstein, M;Gupta, V;Holmes, R;Idarraga, S;James, G;King, T;Klein, T;Kupfer, S;Lebwohl, B;Lowe, J;Murray, J;Newton, E;Quinn, D;Radin, D;Ritter, T;Stacey, H;Strout, C;Stubbs, R;Thackwray, S;Trivedi, V;Tyedin, J;Weber, J;Wilson, S

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