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Evaluating Responses to Gluten Challenge: A Randomized, Double-Blind, 2-Dose Gluten Challenge Trial

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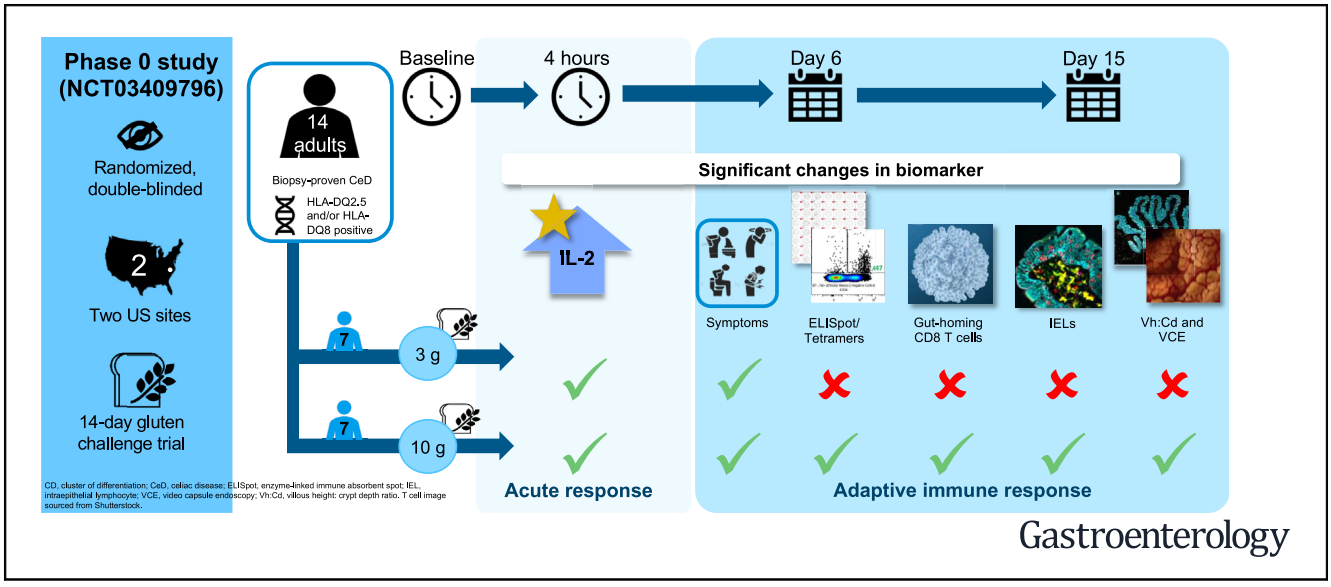

See Covering the Cover synopsis on page 632.

BACKGROUND & AIMS: Gluten challenge is used to diagnose celiac disease (CeD) and for clinical research. Sustained gluten exposure reliably induces histologic changes but is burdensome. We investigated the relative abilities of multiple biomarkers to assess disease activity induced by 2 gluten doses, and aimed to identify biomarkers to supplement or replace histology. **METHODS:** In this randomized, double-blind, 2-dose gluten-challenge trial conducted in 2 US centers (Boston, MA), 14 adults with biopsy-proven CeD were randomized to 3 g or 10 g gluten/d for 14 days. The study was powered to detect changes in villous height to crypt depth, and stopped at planned interim analysis on reaching this end point. Additional end points included gluten-specific cluster of differentiation (CD)4 T-cell analysis with HLA-DQ2-gluten tetramers and enzyme-linked immune absorbent spot, gut-homing CD8 T cells, interleukin-2, symptoms, video capsule endoscopy, intraepithelial leukocytes, and tissue multiplex immunofluorescence. **RESULTS:** All assessments showed changes with gluten challenge. However, time to maximal change, change magnitude, and gluten dose–response relationship varied. Villous height to crypt depth, video capsule endoscopy enteropathy score, enzyme-linked immune absorbent spot, gut-homing CD8 T cells, intraepithelial leukocyte counts, and HLA-DQ2–restricted gluten-specific CD4 T cells showed significant changes from baseline at 10 g gluten only; symptoms were significant at 3 g. Symptoms and plasma interleukin-2 levels increased significantly or near significantly at both doses. Interleukin-2 appeared to be the earliest, most sensitive marker of acute gluten exposure. **CONCLUSIONS:** Modern biomarkers are sensitive and responsive to gluten exposure, potentially allowing less invasive, lower-dose, shorter-duration gluten ingestion. This work provides a preliminary framework for rational design of gluten challenge for CeD research. ClinicalTrials.gov number, NCT03409796.

Keywords: Celiac Disease; Gluten Challenge; Biomarkers; T Cells.

Celiac disease (CeD) is an immune-mediated disorder with estimated global pooled prevalence for biopsy-confirmed CeD at approximately 0.7%.[1] CeD develops in genetically predisposed individuals and is characterized by abnormal T-cell responses to wheat prolamin proteins, predominately gliadins.[2,3] CeD manifestations are heterogeneous and include diarrhea, constipation, abdominal pain, nausea and vomiting, and malabsorption. Systemic manifestations can include infertility, dermatitis herpetiformis, and malignancy.[4,5]

Although gluten avoidance is necessary in CeD, achieving a strict gluten-free diet (GFD) is difficult, because gluten contamination appears to be common when using objective measures of gluten exposure.[6–10] Therefore, many patients attempting to follow a GFD still experience signs and symptoms of active disease. Even when dietary modification results in adequate symptom control, treatment burden is high and patient satisfaction is poor.[11] The inadequacy of a GFD to treat CeD supports the exploration of new therapeutic approaches.[12–17]

Currently, CeD diagnosis and evaluation of therapeutic efficacy are based on patient-reported symptoms, which are subjective; serologic biomarkers, which are not sensitive enough to monitor disease activity; and histologic damage in mucosal biopsies, which requires assessment by a skilled pathologist. Additional less invasive and/or more objective biomarkers of CeD activity have been proposed. Some, such as lactulose to mannitol ratio[18] and intestinal fatty-acid binding protein,[19,20] have shown limited value. Measuring gluten-specific T-cell mobilization into the blood, originally evaluated by enzyme-linked immune absorbent spot (ELISpot),[21] and more recently by whole blood cytokine release assays, and HLA-DQ-gluten tetramers, and gluten-induced cytokines, such as interleukin (IL)-2, have value because they change with gluten exposure.[22–26] Likewise, video capsule endoscopy (VCE) and urine gluten immunogenic peptide (GIPs) have been proposed for monitoring CeD activity and gluten exposure.[27–30] Prior studies have incorporated few of these markers, so it is unclear how they compare in sensitivity, responsiveness, and reliability. Finally, the gluten dose triggering changes across biomarkers is unknown, leading to trials that either fail to demonstrate effects, owing to subthreshold gluten exposure, or that are unnecessarily burdensome due to higher dose and longer duration of gluten exposure than necessary.

To address these issues, a prospective trial in patients with CeD was performed to evaluate the effects of gluten exposure systematically at 3 g and 10 g/d, and to characterize and compare biomarkers measuring these changes. This exploratory study provides integrated measures of response to gluten by patients with CeD, and informs the rational selection of individual biomarkers for future studies.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Histological damage may be used to diagnose and monitor celiac disease (CeD), but it is burdensome for patients. Less invasive, more objective pharmacodynamic biomarkers are required to evaluate CeD activity.

NEW FINDINGS

Of symptoms, Vh:Cd, IEL count, VCE enteropathy score, plasma IL-2 levels, circulating gut-homing CD8, and enumeration of gluten-specific CD4 T cells, change in IL-2 levels appeared the earliest, and was the most sensitive, marker of acute gluten exposure.

LIMITATIONS

This study used a small, demographically homogenous sample of patients with significant intestinal damage before gluten challenge. Results require validation in larger studies and different populations.

IMPACT

This comprehensive assessment of available CeD biomarkers provides a framework for rational design and selection of biomarkers in future gluten-challenge studies, and may inform changes in clinical practice.

Materials and Methods

Study Design

A randomized, double-blind, 2-dose gluten-challenge trial was conducted at Massachusetts General Hospital and Beth Israel Deaconess Medical Center (Boston, MA) during April 2018–May 2019. This study was designed to enroll up to 20 patients, with interim analysis after 12 patients had completed gluten challenge and planned cessation if a statistically significant change from baseline in villous height to crypt depth ratio (Vh:Cd) was observed. The study protocol was approved by the relevant Institutional Review Boards, complied with Good Clinical Practice guidelines, and is registered and accessible at ClinicalTrials.gov (NCT03409796). All authors had access to the study data and reviewed and approved the final manuscript.

HLA-DQ2.5 and/or HLA-DQ8–positive adults with biopsy-proven CeD in clinical and histologic remission on a GFD for $\geq$12 months were enrolled. Patients selected for inclusion had records of diagnostic pathology reviewed at screening, and were required to have no ongoing signs or symptoms that, in the investigator's opinion, were due to CeD. Patients

Abbreviations used in this paper: **CD, cluster of differentiation; CDSD, Celiac Disease Symptom Diary; CeD, celiac disease; CI, confidence interval; ELISpot, enzyme-linked immune absorbent spot; EM, effector memory; FDR, false discovery rate; GFD, gluten-free diet; GIP, gluten immunogenic peptide; HLA, human leukocyte antigen; IEL, intraepithelial lymphocyte; IFN, interferon; IL, interleukin; LP, lamina propria; SFU, spot-forming unit; VCE, video capsule endoscopy; Vh:Cd, villous height to crypt depth ratio.**

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were included if they had a negative urine GIP assay at screening, attested to a GFD, and had near-normal celiac serologies. Exclusion criteria were history of food intolerances/allergy other than to gluten and lactose; severe acute reactions to sporadic gluten ingestion; chronic active gastrointestinal disease other than CeD; or exposure to corticosteroids or other immunosuppressive agents within the prior 3 months.

The study included a 21-day screening period followed by a 7-day run-in period during which patients underwent endoscopy with duodenal biopsy, VCE, and blood collection (Supplementary Figure 1). Run-in was followed by 14-day gluten challenge at the assigned daily dose. Blood biomarkers were measured 4 hours after the first dose (cytokines only), and at days 6 and 15. Endoscopic duodenal biopsy, VCE, and blood collection were repeated after gluten challenge on day 15. A final visit occurred 28 days post-gluten challenge with VCE and blood cytokine assessment. Patients completed the CeD Symptom Diary (CDSD)[14,31] daily from run-in to study end (day 42). Patients underwent serum testing for antibodies to IgA tissue transglutaminase, IgA and IgG deamidated gliadin peptide using QUANTA Lite R h-tissue transglutaminase IgA enzyme-linked immunosorbent assay (INOVA Diagnostics, San Diego, CA) on the BioFlash platform.

Intervention

Patients were block-randomized by site in a 1:1 ratio concurrently to either 3 g gluten/d or 10 g gluten/d for 14 days, using unique randomization sequence numbers and the relevant gluten dose (Supplementary Figure 1). Gluten was administered as Vital Wheat Gluten Flour (Bob's Red Mill, Milwaukie, OR). Gluten concentration was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis[32,33] and the Kjeldahl method (BÜCHI K350, Flawil, Switzerland). Flour protein fraction was 66% of the total flour content and all protein was gluten (3 g dose = 4.5 g flour; 10 g dose = 15 g flour). Gluten doses were supplied in premeasured packets blinded to dose patients. Care providers, the investigator, and outcomes assessor were all blinded after assignment to interventions.

Patients receiving 10 g of gluten could reduce their dose to 3 g after day 3 to address symptoms, with reduction managed by unblinded qualified staff members. Adherence to dosing was monitored using the urine Gluten Detective (GIP) test (Biomedal; iVYDAL, Seville, Spain).[28] This assay detects immunodominant gliadin peptide sequences overlapping with those used in the tetramer and ELISpot assays.

Trial End Points

The primary end point was change in Vh:Cd from baseline to day 15. Secondary and exploratory end points are shown in Supplementary Figure 1.

Upper Endoscopy With Biopsy

Endoscopic duodenal biopsies were obtained using standard disposable biopsy forceps from the second part of the duodenum (D2). Starting distally, 1 biopsy was taken per pass. Four to six biopsies were immediately placed into 10% neutral buffered formalin.

Histology and Morphometry

Each tissue fragment was placed into a separate paraffin block and macroscopically embedded and oriented to allow Vh:Cd evaluation.[34] Hematoxylin- and eosin-stained biopsies were evaluated independently by a blinded gastrointestinal pathologist. On the best oriented profile of each fragment, Vh:Cd was determined by measuring at magnification 100–200× using an eyepiece micrometer. Villous intraepithelial lymphocyte (IEL) infiltration was recorded at magnification 400× as the number of IELs per 100 enterocytes in the field of view in which Vh:Cd was measured; IEL counts were not performed over mucosal lymphoid aggregates.[35] For each endoscopy, results from ≤6 individual biopsies were averaged to produce representative Vh:Cds and IEL counts.[18]

Multiplex Immunofluorescence Staining

Multiplex immunofluorescence[36] was done using the MultiOmyx platform (NeoGenomics Laboratories, Fort Myers, FL) (Supplementary Table 1).

Video Capsule Endoscopy

Patients ingested a commercially available VCE (PillCam SB3 Medtronic, Minneapolis, MN). Extent of villous damage was quantified by a single reader independently reviewing each video (PillCam Web software, version 9.0 US, Build 91.53.20160.0), blinded to patient, time point, and gluten dose. The number of minutes of video with observed villous damage was collected (termed *celiac minutes*).[37]

Tetramer Staining, Enzyme-Linked Immune Absorbent Spot, and Mass Cytometry

Tetramers. DQA1*0501.DQB1*0201 (DQ2) monomers with α-I (QLQPFPQPELPY) or α-II (PQPELPYPQPE) gluten peptides[38] were tetramerized with streptavidin phycoerythrin. $CD4^+$ T cell isolation and staining is described in Supplementary Table 2.

Enzyme-Linked Immune Absorbent Spot. Cryopreserved peripheral blood mononuclear cells were rested overnight, plated at 500,000 cells/well, then either left unstimulated (negative control), stimulated with an αCD3 monoclonal antibody (positive control), or stimulated with a total of 25 μg/mL (12.5 μg/mL of each peptide) of deamidated α-gliadin (QLQPFPQPELPYPQPQS)[39] and deamidated ω-gliadin (PFPQPEQPFPW)[40] peptides, as per instructions for the human interferon (IFN)-gamma ELISpot^{PRO}, MabTech Kit (Nacka Strand, Sweden). Peptides were purchased from JPT Peptide Technologies (Acton, MA; >95% purity). Six replicates were performed for negative controls and peptide-stimulated cells; triplicates were completed for positive controls. Normalized spot-forming unit (SFU) values = mean SFU/million cells from peptide stimulated wells − mean SFU/million cells from negative control wells.

Time-of-Flight Mass Cytometry. Cryopreserved peripheral blood mononuclear cells were stained and subjected to mass cytometry analysis. Staining methods and reagents are described in Supplementary Table 3.

Interleukin-2 Assay. The Single Molecule Array IL-2 2.0 assay and the SIMOA HD-1 Analyzer (Quanterix, Lexington, MA) were used to quantify plasma IL-2 levels.[42] The assay lower limit of detection is 0.1236 pg/mL in plasma.

Statistical Methods. Statistical significance for change from baseline for Vh:Cd was computed using a paired 1-sided Student *t* test. Statistical significance was tested for other changes using a 1-sided Wilcoxon signed-rank test. Changes from baseline for several metrics were correlated using the Spearman's rank correlation.

Sample Size. Sample size was selected to detect changes in Vh:Cd and T-cell markers with 80% power at the interim analysis and >99% by the final analysis, as well as to allow estimation of biomarker differences for secondary and exploratory end points. Using previously published estimates of ΔVh:Cd and Δlog(Tetramer),[18,25] analysis with 12 patients has 92% power to detect a change in Vh:Cd, and 99% power to detect a change in T-cell markers. This was converted to a group sequential design such that interim analysis with 12 patients has 84% power to detect a change in Vh:Cd and 95% power to detect a change in T-cell markers, for an overall power of 80% at interim analysis.

Results

Enrollment

Stopping criteria were met at interim analysis, with significant decrease in Vh:Cd after gluten challenge and significant increase in gluten-specific T cells, and enrollment ceased. Overall, 24 patients were screened and 16 patients were enrolled and randomized (Table 1). Fourteen patients (7 receiving 3 g gluten and 7 receiving 10 g gluten) had 2 endoscopic examinations with biopsies, pre- and post-gluten challenge, with 13 patients completing the 14-day gluten challenge. No patient required a dose reduction. Three patients discontinued owing to gastrointestinal symptoms. A patient in the 10-g group discontinuing gluten challenge at day 10 provided samples at all time points, and the associated data were included for analysis. Demographic and clinical characteristics of both dose groups were similar (Supplementary Table 4); however, more patients in the 3-g than in the 10-g group had gluten exposure before challenge (see below).

Gluten Exposure and Serology

At screening, all patients were negative for urine GIP. Before gluten dosing, 2 patients in the 3-g group were GIP-positive. All patients in the 10-g group and 4 of 7 patients in the 3-g group had detectable GIP on day 6. On day 15, 9 of 14 patients remained GIP-positive (4 patients receiving 3 g, 5 receiving 10 g). On day 42, one patient in the 10-g group was GIP-positive.

Baseline serology was low for all patients. No changes were seen in the 3-g group over 42 days, whereas titers increased in the 10-g group (Supplementary Table 5).

Symptoms

The CDSD is a 6-item, daily symptom scale, including diarrhea, bloating, nausea, abdominal pain, and tiredness. This study included the gastrointestinal domain items of abdominal pain, bloating, nausea, and diarrhea. Gastrointestinal symptoms were low before gluten challenge and increased in both groups during challenge (n = 14; P = .0009), returning to near normal thereafter (Supplementary Figure 2).

Histology

Patients in the 3-g group had a median baseline Vh:Cd (2.0; 95% confidence interval [CI], 1.8–2.4) vs 2.5 (95% CI, 1.6–3.0) for the 10-g group (aggregate: 2.1; 95% CI, 1.6–2.6) (Supplementary Table 4). At day 15, Vh:Cd was 2.1 (95% CI, 1.6–2.6) and 0.6 (95% CI, 0.2–1.3) for the 3-g and 10-g groups (aggregate: 1.4; 95% CI, 0.7–2.1). Median baseline IEL counts were 23.2 (95% CI, 21.1–26.1) and 26.7 (95% CI, 23.1–30.9) for the 3-g and 10-g groups (aggregate: 26.1; 95% CI, 20.6–29.8). At day 15, IEL counts had increased in both groups, with a median of 40.2 (95% CI, 28.8–42.9) in patients receiving 3 g gluten and of 54.2 (95% CI, 46.3–63.5) in those receiving 10 g gluten (aggregate: 42.9; 95% CI, 34.8–53.9). There was significant change in the aggregated Vh:Cd (P = .0044) and IEL counts (P = .0026; Figure 1). A nonsignificant change was observed in the 3-g group for Vh:Cd (P = .23) and IEL counts (P = .15). Conversely, in the 10-g group, 6 of 7 patients had substantial reductions in Vh:Cd (P = .0025), and all patients had increased IEL counts (P = .0078).

Video Capsule Endoscopy

Both groups showed minimal damage at run-in (Figure 2). Celiac minutes of enteropathy, collected as described previously,[37] increased from baseline but not significantly for the group overall (P = .14). The increase reached significance in the 10-g group (P = .047) vs. in the 3-g group (P = .74; representative images in Supplementary Figure 3). All patients experiencing increase in celiac minutes in response to gluten had a decrease in celiac minutes by day 42, except for 1 patient in the 10-g group who exhibited a delayed increase in celiac minutes from baseline, demonstrating maximal number of celiac minutes at day 42. As expected, villous damage was more severe proximally (J. Siegelman et al, unpublished data, May 2020).

Gluten-Specific Peripheral Blood T Cells

IFN-gamma SFUs were negligible during run-in, increased significantly at day 6, and returned to near baseline levels by day 15 (Figure 3*A*; overall change P = .003). Only 1 patient in the 3-g group had a positive response (≥10 SFUs per 10^6 peripheral blood mononuclear cells). This patient also had increases in tetramer-positive cells and IL-2 (Table 1), but no clear effect on Vh:Cd. In contrast, the 10-g group showed significant change from baseline in IFN-gamma SFUs (P = .016). All patients in the 10-g group with positive IFN-gamma SFU response (4 of 7 patients) had a reduction in Vh:Cd ≥1.

HLA-DQ-gluten tetramer staining, activated ($CD38^+$), EM ($CD45RA^-$, $CD62L^-$), gut-homing ($\beta7^+$) CD4 T cells were also quantified.[38] Overall, significant increases were seen in HLA-DQ2 gluten-specific CD4 T cells from

Table 1. Patient Characteristics and Biomarker Responses for All Patients, Including Those Discontinuing Gluten Challenge

Patient characteristics and treatment					Change from baseline to peak response[a]			
Patient no.	HLA allele 1	HLA allele 2	Category	Gluten dose, *g*	Tetramer[b] (fold-change)	ELISpot[b] (fold-change)	IL–2[c] (fold-change)	Vh:Cd[d] (absolute difference)
1[e]	DQ2.5	B1.02	Homozygous DQ2	3	NA	NA	>100	NA
2	B1.02	—	Other	3	2–10	No change	10–100	No change
3	B1.02	—	Other	10	No change	No change	10–100	No change
4	DQ2.5	—	Heterozygous DQ2	10	2–10	No change	>100	>1.0
5	DQ2.5	DQ2.5	Homozygous DQ2	10	>100	2–10	>100	>1.0
6	DQ2.5	—	Heterozygous DQ2	3	2–10	>100	10–100	No change
7	DQ8	—	DQ8	3	No change	No change	No change	0.4–1.0
8	DQ8	B1.02	Other	10	No change[f]	2–10	>100	>1.0
9	DQ2.5	—	Heterozygous DQ2	10	>100	10–100	10–100	>1.0
10	DQ8	—	DQ8	3	No change	No change	No change	No change
11	DQ2.5	—	Heterozygous DQ2	3	No change	No change	10–100	No change
12	DQ2.5	DQ2.5	Homozygous DQ2	10	>10–100	10–100	10–100	>1.0
13	DQ2.5	DQ2.5	Homozygous DQ2	10	2–10	No change	>100	>1.0
14[e]	DQ2.5	DQ2.5	Homozygous DQ2	3	NA	NA	>100	NA
15	DQ2.5	DQ8	Heterozygous DQ2 + DQ8	3	2–10	No change	>100	0.4–1.0
16	DQ2.5	—	Heterozygous DQ2	3	No change	No change	2–10	No change

NA, not applicable.
[a]Change from baseline (highest baseline value either at run-in or day 0) to peak response.
[b]Fold increase from baseline, peak response at day 6.
[c]Fold increase from baseline, peak response at day 1, 4 hours post gluten ingestion.
[d]Actual reduction from baseline, peak response at day 15.
[e]Patient discontinued gluten challenge.
[f]Run-in used as baseline comparator.

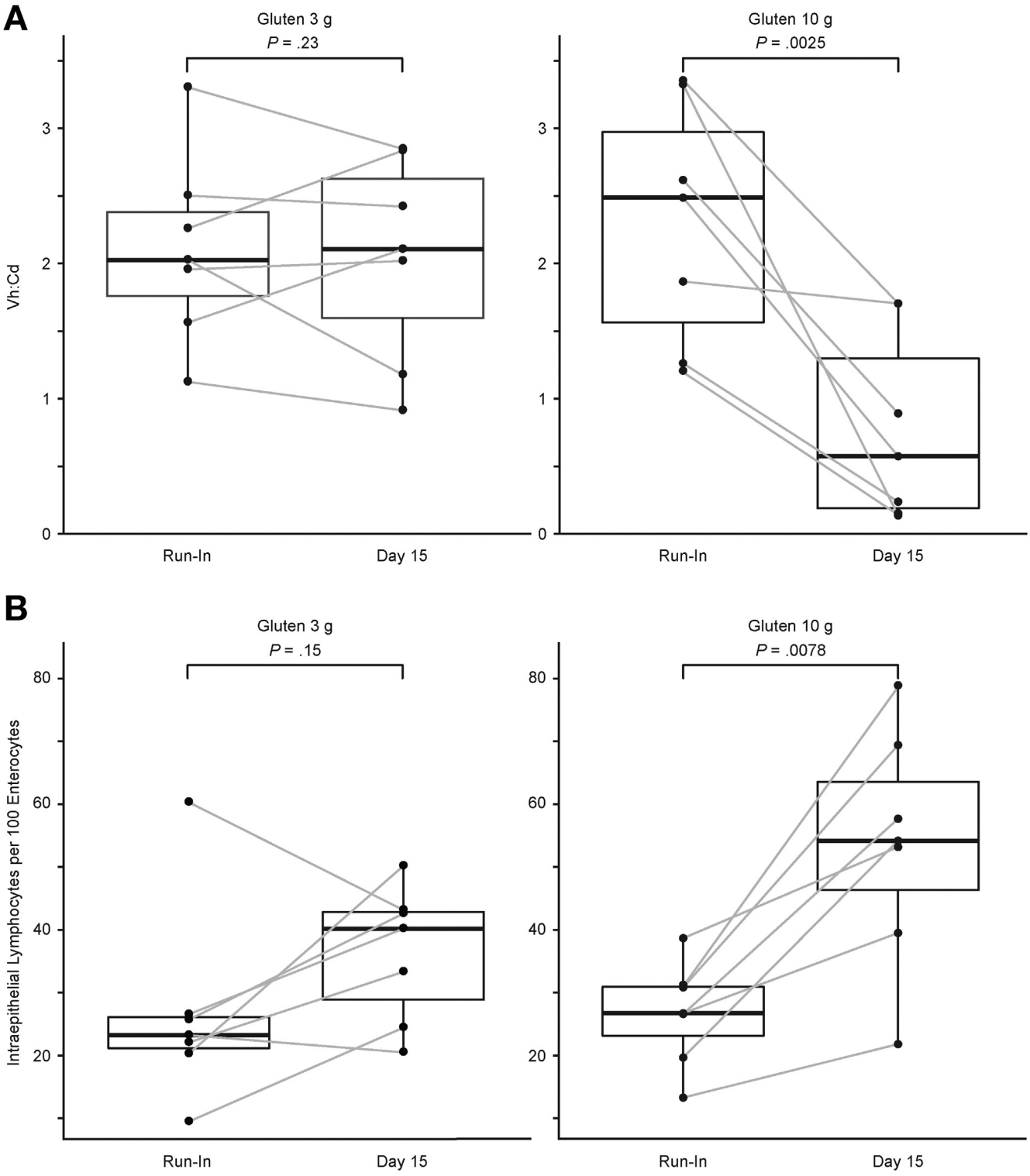

Figure 1. Change in Vh:Cd and IEL count after gluten challenge. Change in duodenal mucosal biopsy Vh:Cd (*A*) and IEL count (*B*) after 3 g or 10 g gluten. *Boxplots* represent medians and quartiles, and *lines* between data points connect results from individual patients. *P* values calculated using paired 1-sided Student *t* test for Vh:Cd and 1-sided Wilcoxon signed-rank test for IEL count. GC, gluten challenge.

baseline on day 6 of gluten dosing (Figure 3*B*; $P = .005$). In the 3-g group, 3 of 7 patients had moderate increases (2.7-fold, 3.2-fold, and 4.4-fold) in tetramer-positive T cells. One of these patients also had an increase in IFN-gamma SFUs at day 6 (described above). In the 10-g group, significant overall increase was observed in HLA-DQ2 gluten-specific CD4 T cells ($P = .016$). For the 2 of 7 patients in the 10-g group without tetramer

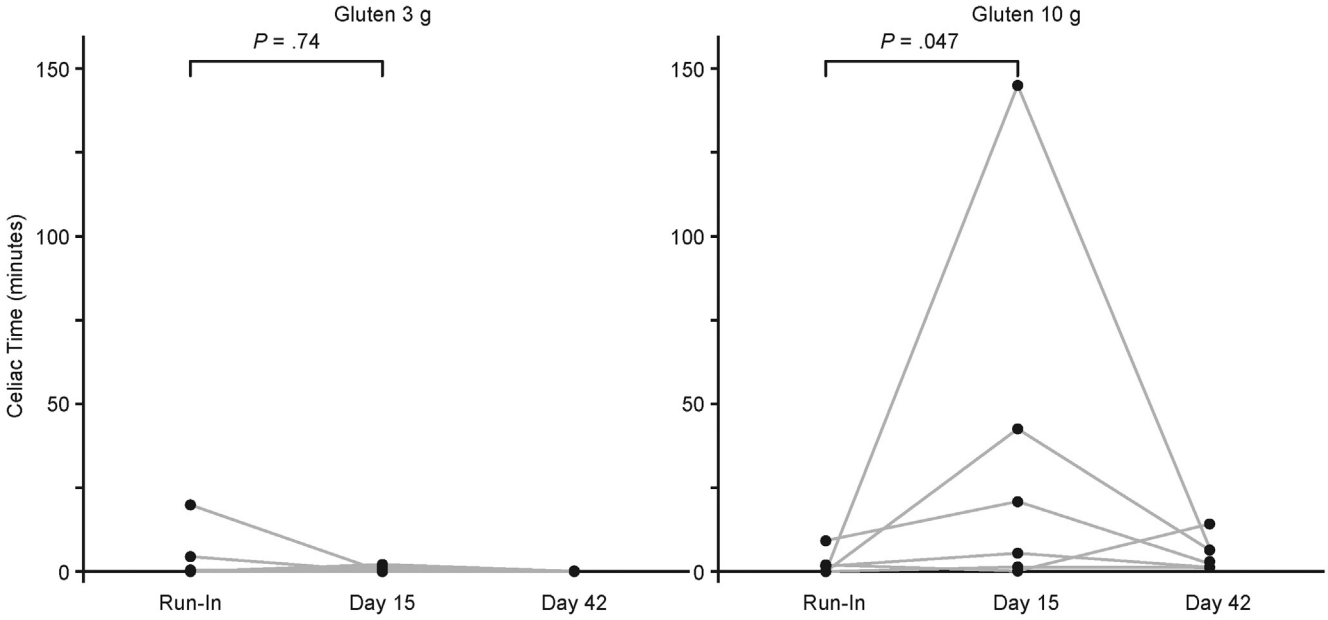

Figure 2. Characterization of small intestine damage by VCE. Small intestine changes detected by VCE in patients receiving 3 g or 10 g gluten. *Lines* between data points connect results from individual patients. *P* values calculated using 1-sided Wilcoxon signed-rank test. GC, gluten challenge.

increases, one was DQ8 positive and the other expressed only DQB1*02 (Table 1).

T-Cell Changes in Blood and Tissue

Gut-homing CD8+ T cells. The percentage of activated ($CD38^+$), gut-homing ($\alpha4^+$, $\beta7^+$), and EM ($CCR7^-$ $CD45RA^-$) CD8 T cells was low in peripheral blood mononuclear cells from patients with CeD before gluten challenge (0–0.88% of $CD8^+$ EM T cells; Figure 4). Change from baseline in gut-homing EM CD8 T cells increased at day 6 (both groups, $P = .0044$), with preferential responses in patients receiving 10 g of gluten ($P = .016$) vs 3 g of gluten ($P = .14$).

Lamina propria and epithelial T cells. Multiplex immunofluorescence (Supplementary Figure 4) revealed $CD3^+$ CD8 T cells (489 cells/mm^2) and CD4 T cells (643 cells/mm^2) were present in biopsies at baseline (mean, n = 13). Both cell types were in the lamina propria (LP) and epithelium, but CD8 T cells had similar levels in both compartments before gluten challenge (LP, 439 cells/mm^2 vs epithelium, 524 cells/mm^2), whereas CD4 T cells were localized in the LP (LP, 1149 cells/mm^2 vs epithelium, 95 cells/mm^2). Some CD8 T cells (37%) had a memory phenotype ($CD45RO^+$; 183 cells/mm^2) and were present in both compartments (LP, 205 cells/mm^2; epithelium, 154 cells/mm^2). Likewise, the majority of CD4 T cells in the LP (68%) had a memory phenotype (LP $CD45RO^+$; 777 cells/mm^2). Based on Ki67 expression, few memory CD8 T cells were proliferating in either compartment (LP, 17 cells/mm^2 vs epithelium, 33 cells/mm^2) and few memory CD4 T cells were proliferating in the LP (34 cells/mm^2).

On day 15, there was an increase in CD8 T cells of 1.5-fold (range, 0.75- to 2.27-fold) at the 3-g dose and 1.6-fold (range, 0.86- to 3.18-fold) at 10 g. In patients receiving 10 g, the greater change was seen in memory CD8 T cells in the epithelium (4.9-fold) vs LP (1.5-fold). Proliferating ($Ki67^+$) memory CD8 T cells in the epithelium were increased 27.1-fold vs proliferating cells with the same phenotype in the LP (increased 8.8-fold). The 3-g gluten challenge showed an appreciable increase in $Ki67^+$ memory CD8 T cells (12.7-fold) vs baseline, primarily due to large increases in $Ki67^+$ memory T cells in 2 of 6 patients (60.5-fold and 11.5-fold), but no enrichment in the LP or epithelium.

Gluten challenge also increased the number of CD4 T cells, albeit more modestly than CD8 T cells. There was no overall increase in CD4 T cells at 3 g gluten and a 1.3-fold increase (range, 0.65–1.94-fold) at 10 g gluten. Memory CD4 T cells showed a similar pattern and increased slightly post-gluten challenge, both in the LP and epithelium. Proliferating memory $CD3^+$ CD4 T cells were seen at both 3 g (3.5-fold) and 10 g (4.9-fold) doses in the LP. In all but 1 patient, the number of proliferating memory $CD3^+$ CD4 T cells in the epithelium was very low ($\leq$25 cells/mm^2) after gluten challenge.

Changes in Interleukin-2

All patients had levels of IL-2 <1 pg/mL before gluten challenge (Figure 5). Four hours after challenge, IL-2 levels increased ($P = .0008$) in 12 of 14 patients, with individual IL-2 levels ranging from 000.30 to 348.04 pg/mL. By day 6, IL-2 levels had declined to near-baseline levels, and to baseline levels at day 15. All patients receiving 10 g of gluten showed increases in IL-2. Patients receiving 3 g of gluten had changes of lesser magnitude and 2 $HLA\text{-}DQ8^+$ patients showed little increase (Table 1).

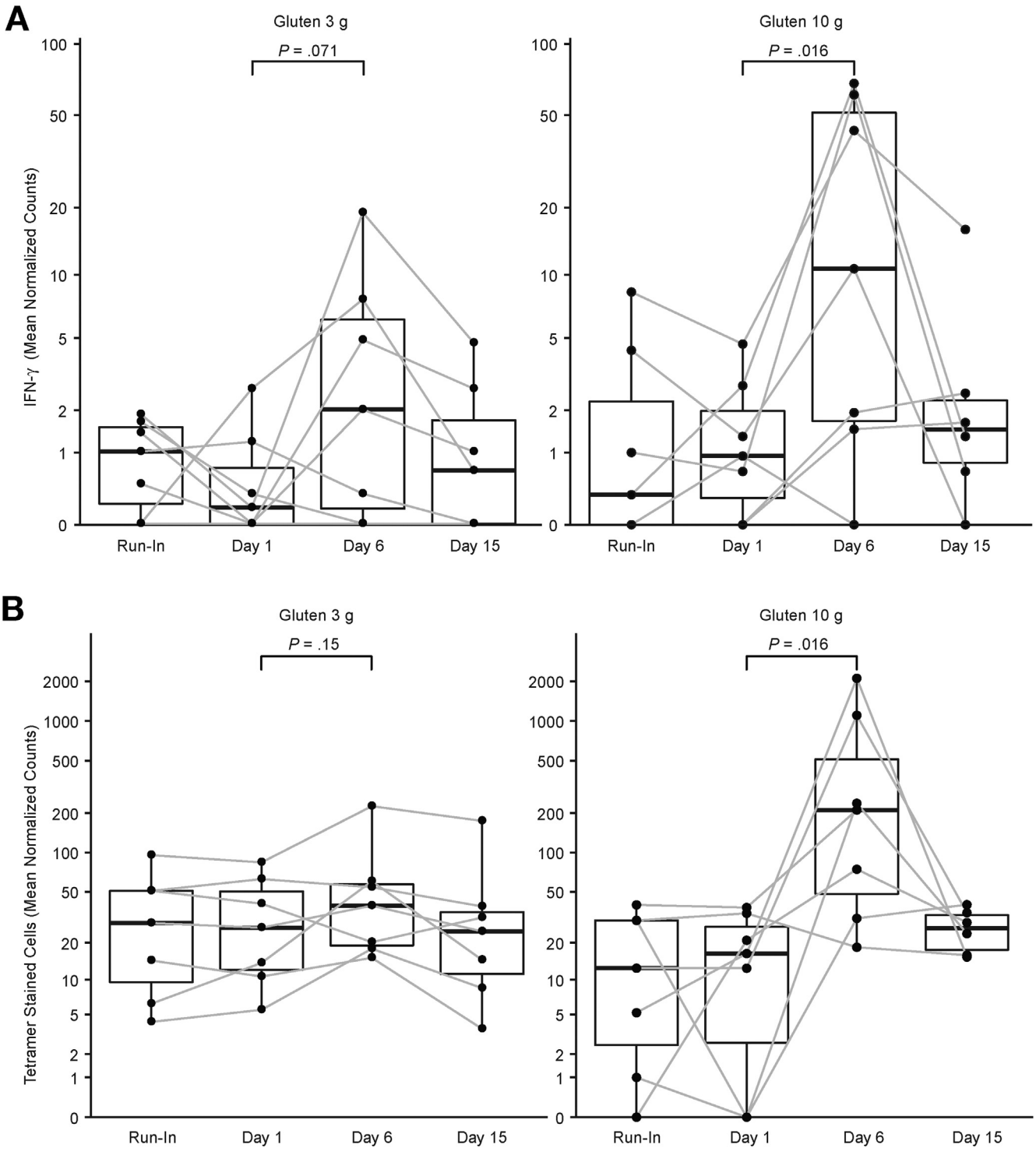

Figure 3. Change in gluten-specific T cells. Change in gluten-specific T cells measured by IFN-gamma ELISpot (*A*) or HLA-DQ2-gluten tetramer-positive, CD38$^+$ $\beta7^+$ EM CD4 T cells (*B*). *Lines* between data points connect data from individual patients. Day 1 and day 6 results were compared, and *P* values calculated using 1-sided Wilcoxon signed-rank test. One patient (3 g gluten) lacked a day-6 sample for ELISpot analysis. GC, gluten challenge.

Data Integration

The kinetics and magnitude of biomarker expression after gluten challenge in patients with CeD are distinct. Four hours after initial challenge, increased plasma IL-2 levels were detected. Elevations of IL-2 were seen in 86% of patients (7 of 7 patients [100%] in the 10-g group vs 5 of 7 [71%] in the 3-g group). Symptoms recorded before gluten challenge showed 57% of all patients reporting a 2-fold increase in CDSD symptom score (with a score >1) during gluten challenge; 3 of 7 (43%) in the 10-g group, and 5 of 7

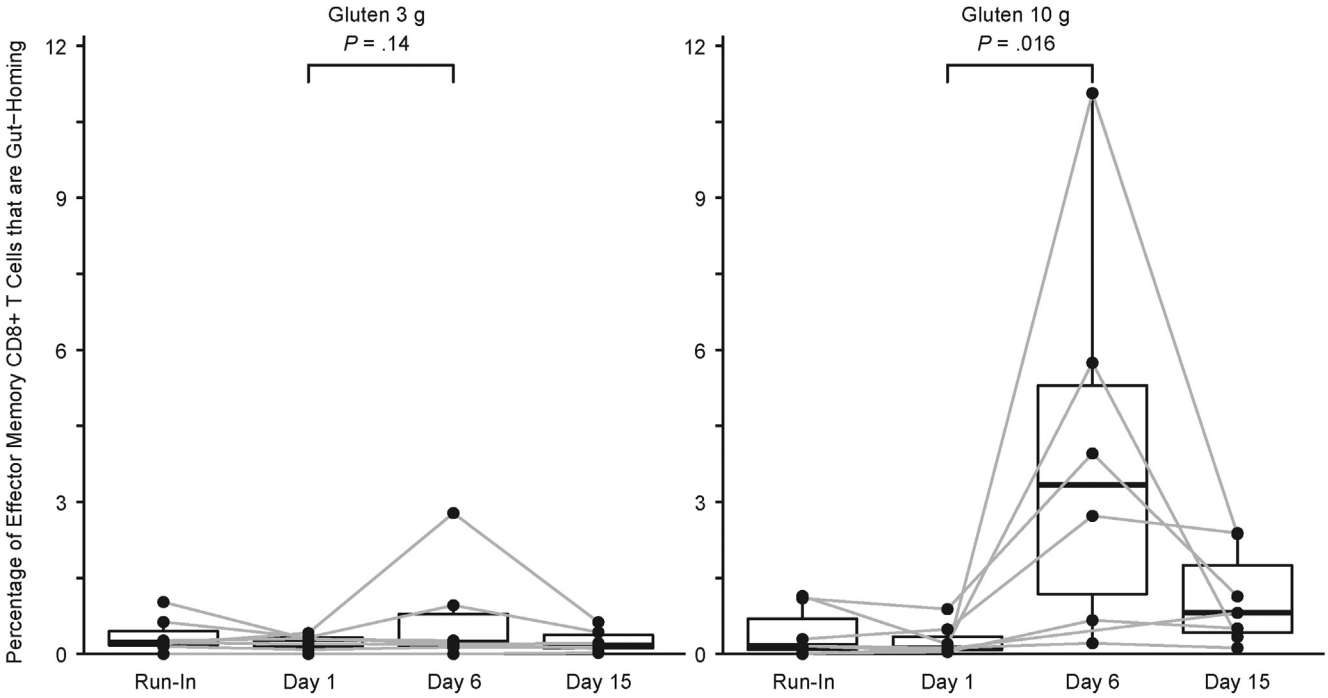

Figure 4. Change in gut-homing CD8$^+$ T cells. Gut-homing EM CD8$^+$ T cells express α4, β7 and are CD38 bright. *Boxplots* represent means and the *lines* between data points connect results from individual patients (n = 12). Day 1 pre dose and day 6 results were compared, and *P* values calculated by 1-sided Wilcoxon signed-rank test. Two patients (1 receiving 3 g and 1 receiving 10 g of gluten) lacked day-6 samples and were not included in this analysis. GC, gluten challenge.

(71%) in the 3-g group. Timing of the increase varied, with highest daily scores recorded anytime between day 1 and day 39.

By 6 days post-gluten challenge, gluten-specific T cells and EM gut-homing CD8 T cells were increased. HLA-DQ-gluten tetramer-positive CD4 T cells were elevated more than 2-fold over baseline in 57% of patients (6 of 7 patients [86%] in the 10-g group vs 2 of 7 patients [29%] in the 3-g group). IFN-gamma–secreting gluten-specific T cells were elevated in 36% of patients (4 of 7 patients [57%] in the 10-g group vs 1 of 7 patients [14%] in the 3-g group), and EM gut-homing CD8 T cells were elevated more than 2-fold in 50% of all patients (5 of 6 patients in the 10-g group and 1 of 6 patients in the 3-g group).

At day 15, gluten-specific T-cell and EM gut-homing CD8 T-cell levels were near normal, whereas intestinal damage was evident in most patients receiving 10 g of gluten. Vh:Cd was reduced by $\geq$1 in 43% of patients (6 of 7 [86%] in the 10-g group vs 0 of 7 in the 3-g group). Intestinal damage, measured by VCE, was increased in 62% of patients (5 of 6 [83%] in the 10-g group vs 3 of 7 [43%] in the 3-g group). IEL counts were increased 2-fold in 43% of patients (4 of 7 patients [57%] receiving 10 g of gluten vs 2 of 7 [29%] receiving 3 g of gluten). Proliferating memory CD8 T cells in the epithelium were increased at least 2-fold in 54% of patients overall (5 of 7 patients [71%] treated with 10 g of gluten vs 2 of 6 [33%] in the 3-g group).

At day 42, VCE, symptoms, and IL-2 were tested. IL-2 had returned to baseline levels; but in 1 patient (10 g of gluten), the number of observed celiac minutes of VCE was greatest at day 42.

Exploratory analysis of the correlation between the maximum change from baseline of different biomarkers was performed to identify potential relationships between individual markers (Figure 6; Supplementary Table 6). After accounting for multiple comparisons (false discovery rate [FDR] = 0.1), the relationship between IL-2 and changes in IEL counts, when all groups were pooled (r = 0.83; P = .0004, FDR = 0.018), was significant. Similar apparent correlations were seen in both the 3-g and 10-g dose groups, with r = 0.93 and r = 0.71, although neither subgroup met the FDR threshold. Other relationships did not have as strong a correlation or meet the FDR, and/or lacked consistency across dose groups, likely owing to the low number of patients tested combined with the limited ability of 3 g of gluten to induce change.

Discussion

Several methodologies have recently been developed that permit broader and more sensitive assessment of intestinal and circulating biomarkers that might lead to novel insights into CeD. In this study, we evaluated traditional and newly described techniques as pharmacodynamic tools to measure response to 2 levels of gluten exposure in CeD. Some assays, such as the tetramer-based assays, were modified from the original description to minimize variability due to complex enrichment protocols and to optimize the number of tetramers used.[25,43] Consistent with prior

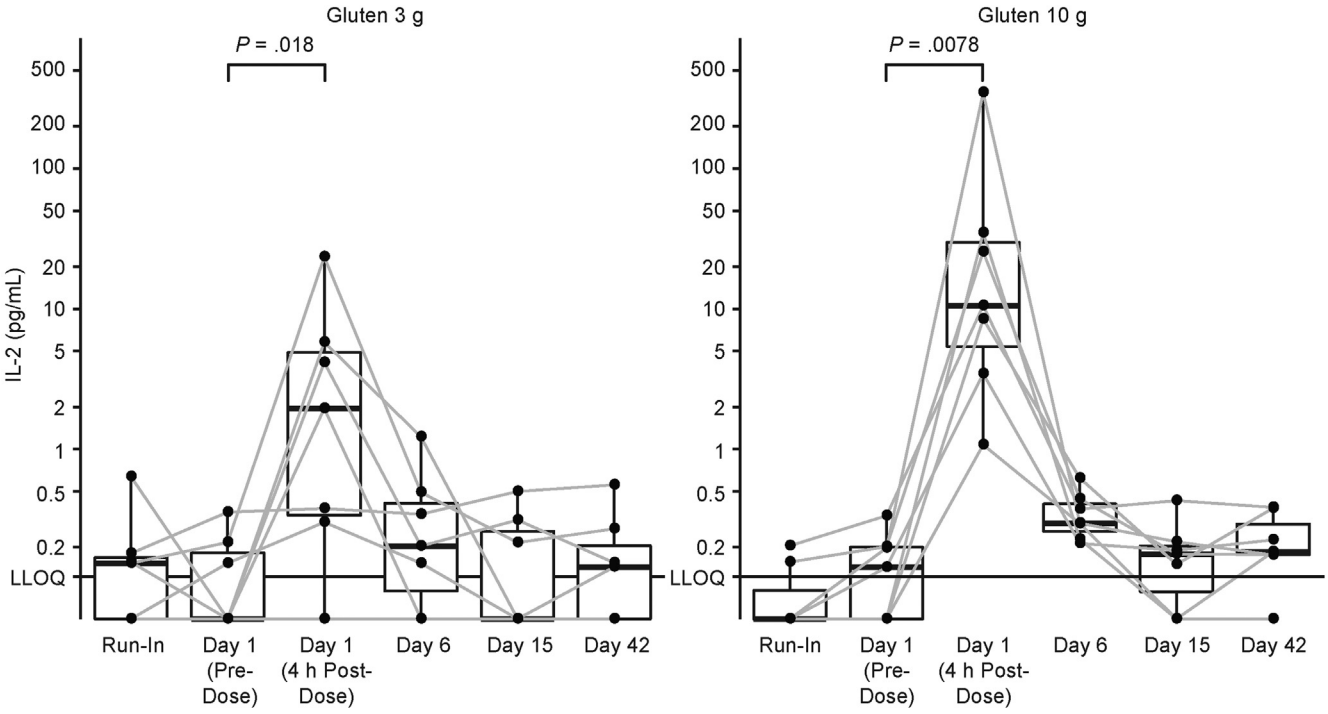

Figure 5. Change in plasma IL-2. *Boxplots* represent means. *Lines* between data points connect data from individual patients. Day 1 pre-dose and post-dose results were compared, and *P* values calculated by 1-sided Wilcoxon signed-rank test. GC, gluten challenge; LLOQ, lower limit of quantification.

studies, symptoms and histology had high inter-individual variability, as exemplified by the CDSD (symptoms), or limited dynamic range and sensitivity, as seen with Vh:Cd (histology). Variation in detectable urine GIP was also observed, likely due to differences in the time of gluten excretion, urine collection, and patient technique. Every biomarker assessed had gluten dose-dependent and time-dependent responses. This biomarker spectrum allows for tailoring of studies for specific clinical and research questions.

The current focus in translational medicine is to develop blood-based, minimally invasive biomarkers. However, direct evaluation of the target organ in CeD, the small intestine, is possible and provides important information. Vh:Cd and IEL count focus on specific, microscopic changes, allowing investigators to judge villous blunting and lymphocyte infiltration.[35,44] VCE takes a broader look at the total burden of macroscopic damage from duodenum to ileum by capturing images throughout the small intestine.[37] Assessing the entire small intestine can address questions about the extent of damage, including areas inaccessible to endoscopy. This analysis showed that VCE, like Vh:Cd, even in a short-duration gluten challenge, allows quantification of damage.

In patients with CeD, gluten-activated T cells populate both the epithelium and LP and contribute to intestinal mucosa damage.[44] We demonstrated that $CD8^+$ memory T cells are present in both the LP and epithelium in patients with CeD at baseline, but after gluten challenge, epithelial $CD8^+$ cell numbers increased and a substantial number of $CD8^+$ T cells expressed Ki67, consistent with a proliferating phenotype. Change in the number of $Ki67^+$ CD8 T cells was dramatic and could offer a disease-relevant pharmacodynamic biomarker, particularly for evaluating therapies targeting T-cell–mediated epithelial cell damage.

In this study, ELISpot and tetramers increased from baseline at day 6. ELISpot has been established as a blood-based approach to monitor gluten response.[21,39] Tetramer studies have confirmed that gluten-specific T cells are $CD4^+$ gut-homing memory T effector cells, and that the number of gluten-reactive T cells in patients with CeD positively correlated with the degree of histologic damage.[25,45] More recently, an increase in a subset of gut-homing activated CD8 and CD4 EM T cells was shown to correlate with similar cell phenotypes in duodenal biopsies after gluten challenge has been described.[24,46]

Using mass cytometry, we found a significant increase of the CD8 gut-homing EM T-cell subset in blood after gluten challenge. This approach was nearly as sensitive at detecting gluten exposure as ELISpot and tetramer staining. Quantification of gut-homing CD8 T cells offers practical advantages over quantifying antigen-specific T cells. EM CD8 gut-homing T cells are more plentiful than antigen-specific T cells and assessment does not require large blood volumes. Approximately 35 mL of blood was collected for each gluten-specific T-cell assay to ensure that there were enough cells to reliably detect the low number of antigen-specific cells. In contrast, for evaluating gut-homing EM CD8 T cells (as well as several other cell types), only

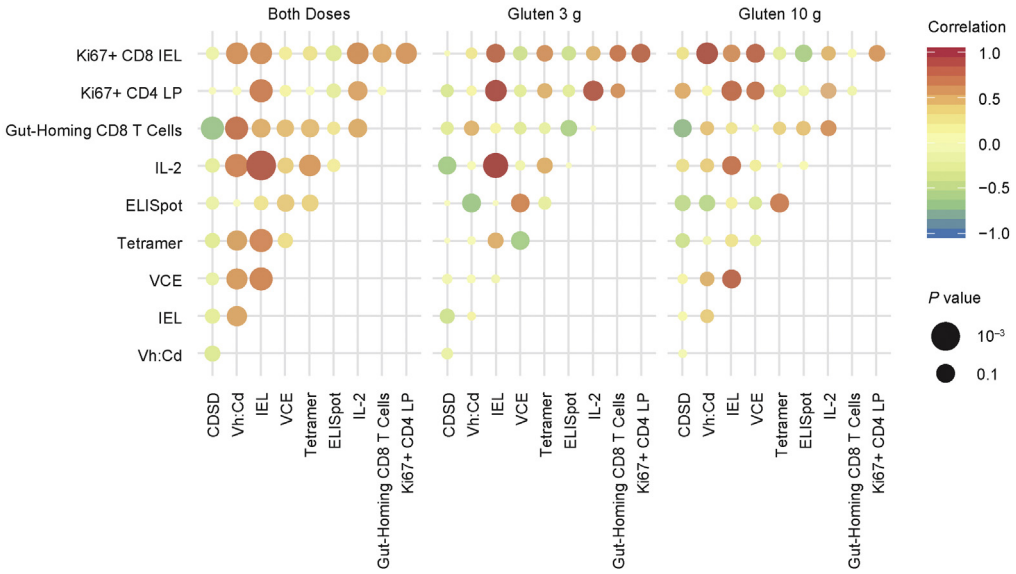

Figure 6. Data integration. Biomarker correlations. Maximum biomarker response was compared with both doses pooled and at each gluten dose by Spearman correlation. Vh:Cd change was inverted before calculating correlation because decreasing Vh:Cd signifies increasing severity, while for other markers increasing score reflects increasing severity.

approximately 5 mL of blood is needed, with no prerequisite and no in vitro culture or enrichment. Furthermore, this technique is scalable and feasible in both research and clinical settings.

Gluten-specific CD4 T cells, by virtue of gliadin specificity, arise as a direct result of gluten exposure and are nearly unique to patients with CeD.[43] Significant changes in these cells after therapeutic intervention provides a clear marker of impact on CeD pathophysiology. Conversely, although a promising biomarker, the role of CD8 gut-homing T cells in the pathology of CeD is still unclear.[24]

After gluten challenge, IL-2 increases rapidly in patients with CeD, but not in healthy controls. The increase is associated with symptom severity and is one of the earliest and most dynamic soluble blood biomarkers of gluten exposure to date; therefore, we chose to focus on this cytokine.[47,48] IL-2 is an acute measure of gluten response with increases observed 4 hours after exposure, and requires only a single-dose gluten challenge and minimal volumes of plasma ($<$0.5 mL blood). We detected an IL-2 response at both 3 g and 10 g of gluten. This sensitivity was possible in part through use of newer assays with high sensitivity and expanded dynamic range. This methodology is feasible for large studies, and further reduces patient burden in terms of gluten exposure and blood sampling. As with gut-homing $CD8^+$ memory cells, the relationship of IL-2 to disease pathology is unclear; however, based on its gluten-specific induction in CeD and expression primarily by activated T cells, it may be important in disease processes.[49] Two patients who were HLA-DQ8 heterozygous did not show increases in IL-2 on gluten exposure. It is tempting to link the absence of HLA-DQ2 to the lack of IL-2 expression; however, it is possible that with a higher dose of gluten these patients could respond. Overall, IL-2 as a biomarker provides the potential for less-invasive, lower-dose, and shorter-duration gluten ingestion, although more research is necessary.

Variation seen in biomarker response to gluten likely reflects the biologic requirements to achieve the change reflected by each biomarker. The biomarkers evaluated measure different and time-sequentially established CeD processes—acute response to gluten exposure (IL-2), gluten-specific CD4 T-cell activation and T-cell trafficking to the intestine, gluten-mediated inflammatory response in the small intestine (increases and phenotypic changes in IELs), and epithelial damage (Vh:Cd). For example, increases in IL-2 on gluten exposure appear to be a relatively sensitive early biomarker of acute gluten exposure in most patients, whereas intestinal damage is a more complex downstream end point with multiple variables potentially impacting Vh:Cd, meaning longer-duration and higher-dose gluten exposure are necessary for changes to be reliably elicited. The discrepancy between serologic markers and recorded enteropathy has been described previously.[50,51] Prior studies have reported that high-dose gluten exposure is not required to induce epithelial damage.[18,52,53] In our study, limited changes in Vh:Cd after a 14-day challenge with 3 g of gluten were observed, in accordance with Sarna et al.[25] Although the 3-g dose was sufficient to initiate an immune response, as detected by several biomarkers, such as IL-2, the 10-g dose was required for enteropathy within the study time frame. Based on our data, we would suggest that gluten challenge should be conducted over longer durations and/or using doses of gluten of $\geq$3 g/d to ensure sufficient histologic change can be induced.

The major limitation of this study, the relatively small sample size, is partially due to the primary end point being met at interim analysis. In addition, the study population was demographically homogeneous and had a significant amount of intestinal damage before gluten challenge, despite stringent inclusion criteria. These issues highlight that confirmation of these results in other populations is important, including patients with better-treated CeD, those displaying non-DQ2.5 genotypes, or children; nevertheless,

our results are largely consistent with prior studies[18,25,27] and we have demonstrated new elements, such as investigation of IL-2, that represent significant advances in the field.

Importantly, the biomarkers described could be used in the clinic as well as in research. For example, evaluation of potential CeD in patients on a GFD could begin with HLA typing followed by, in patients with permissive genetics, single-dose gluten challenge with IL-2 measured 4 hours post gluten challenge.[47,54] Patients without IL-2 response are unlikely to have CeD, and further testing is not required.[47,54] If IL-2 response is positive, confirmatory testing could be performed, either with gluten-specific T-cell response on day 6, or histologic assessment after $\geq$14 days of gluten exposure, optimally with high-dose gluten if tolerated.

In research, single-dose gluten challenge with IL-2 response can be used to confirm veracity of CeD diagnosis before trial enrollment. To reduce patient burden, early studies should assess prevention of IL-2 response and gluten-specific T-cell response after 1 and 6 days, respectively. If an intervention cannot modify these responses, disease modification is unlikely and further studies might not be warranted; however, positive data based on cytokine and T-cell response should be confirmed with assessment of small intestinal mucosal injury, at least in a subset of patients. Together, these recommendations have the potential to use novel tests based on the known pathophysiology of CeD, thereby improving efficiency and reducing the burden of both clinical care and research. Once validated, approaches described here might replace more traditional histologic methods of diagnosing and defining CeD.

In conclusion, this study provides a comprehensive assessment of CeD biomarkers and performance in gluten challenge across 2 commonly used gluten doses, and underscores the challenges of diagnosing CeD and monitoring therapy. Selected CeD biomarkers are sensitive and responsive to gluten exposure, providing the potential for less-invasive, lower-dose, and shorter-duration gluten ingestion. These data, along with prior studies,[13,18,25,48] provide a framework for rational design and selection of biomarkers in future gluten-challenge studies, and can inform changes in clinical practice.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2020.10.040.

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Conflicts of interest
The authors disclose the following: Maureen M. Leonard has a speaker agreement with Takeda Pharmaceuticals, sponsored research with Glutenostics LLC, and is a consultant to Anokion and HealthMode Inc. Jocelyn A. Silvester has served on an advisory board for Takeda Pharmaceuticals and has received research funding from Biomedal S.L., Cour Pharmaceuticals, and Glutenostics LLC. Her salary is supported by the National Institutes of Health under award DK K23 119584. Alessio Fasano is a co-founder and stock holder of Alba Therapeutics, has a speaker agreement with Mead Johnson Nutrition, and is a member of NextCure and Viome SAB. Ciarán P. Kelly has been a scientific consultant to Cour Pharmaceuticals, Glutenostics, ImmunogenX, Innovate, and Takeda, is a stock grantee of Cour and Glutenostics, and is an investigator for Aptalis, ImmunogenX, Innovate, and Takeda. Suzanne K. Lewis has been a consultant to Invicro and Takeda Pharmaceuticals. Jeffrey D. Goldsmith has been a consultant to Merck Research Laboratories and Takeda Pharmaceuticals. Elliot Greenblatt works for Invicro, A Konica Minolta Company, which was engaged by Takeda to develop quantitative analyses of capsule endoscopy. William W. Kwok and I-Ting Chow are full-time employees of Benaroya Research Institute at Virginia Mason. Daniel Leffler, William J. McAuliffe, Kevin Galinsky, Jenifer Siegelman, and Glennda Smithson are full-time employees of Takeda Pharmaceuticals Inc. Co. John A. Wagner and Anna Sapone were full-time employees of Takeda Pharmaceuticals Inc. Co. at the time of this study.

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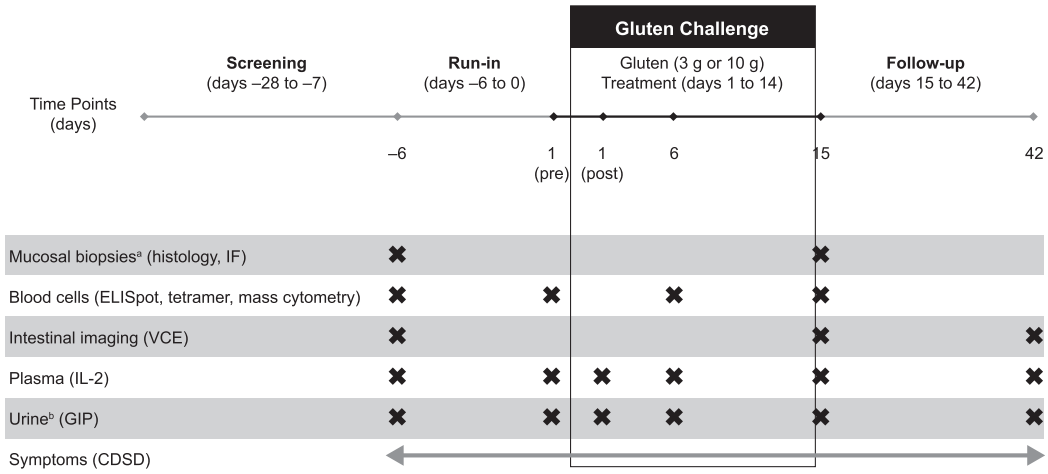
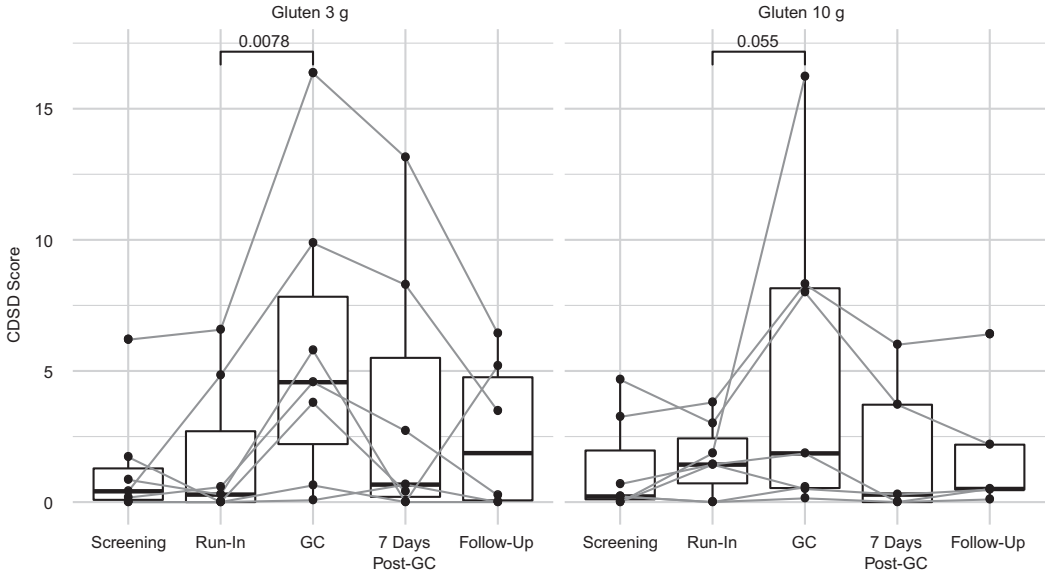
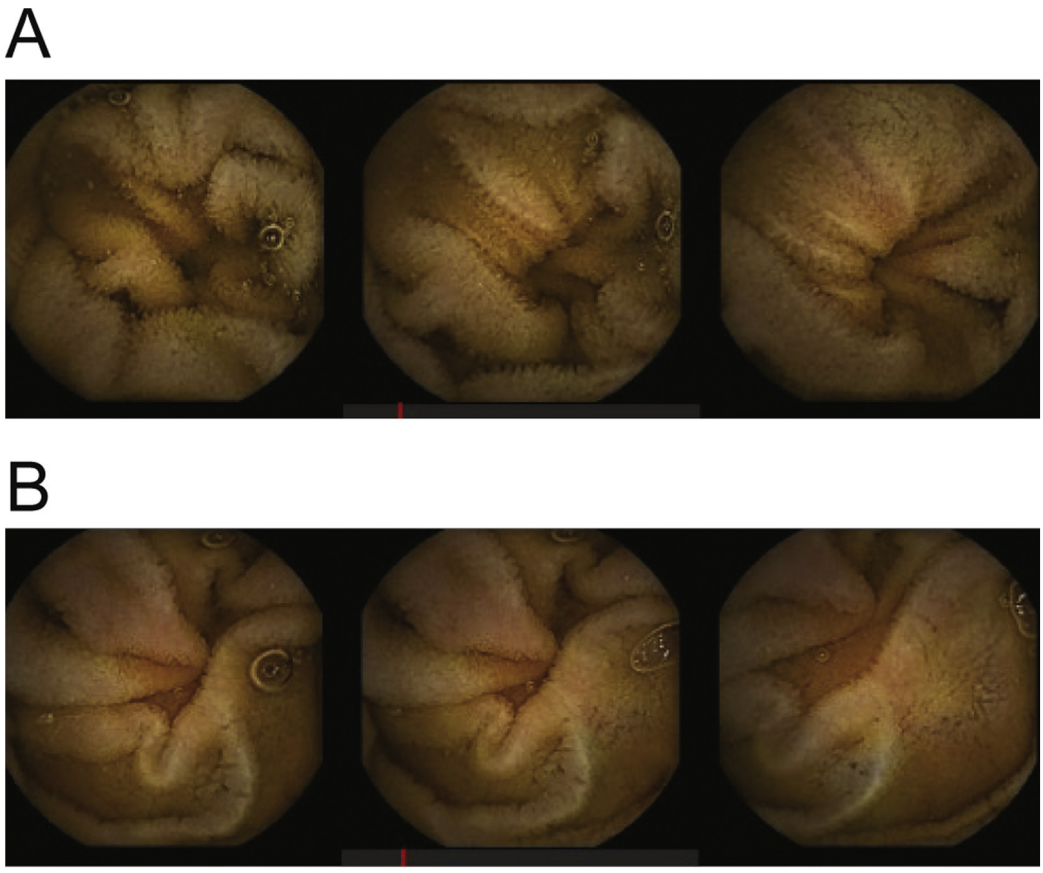


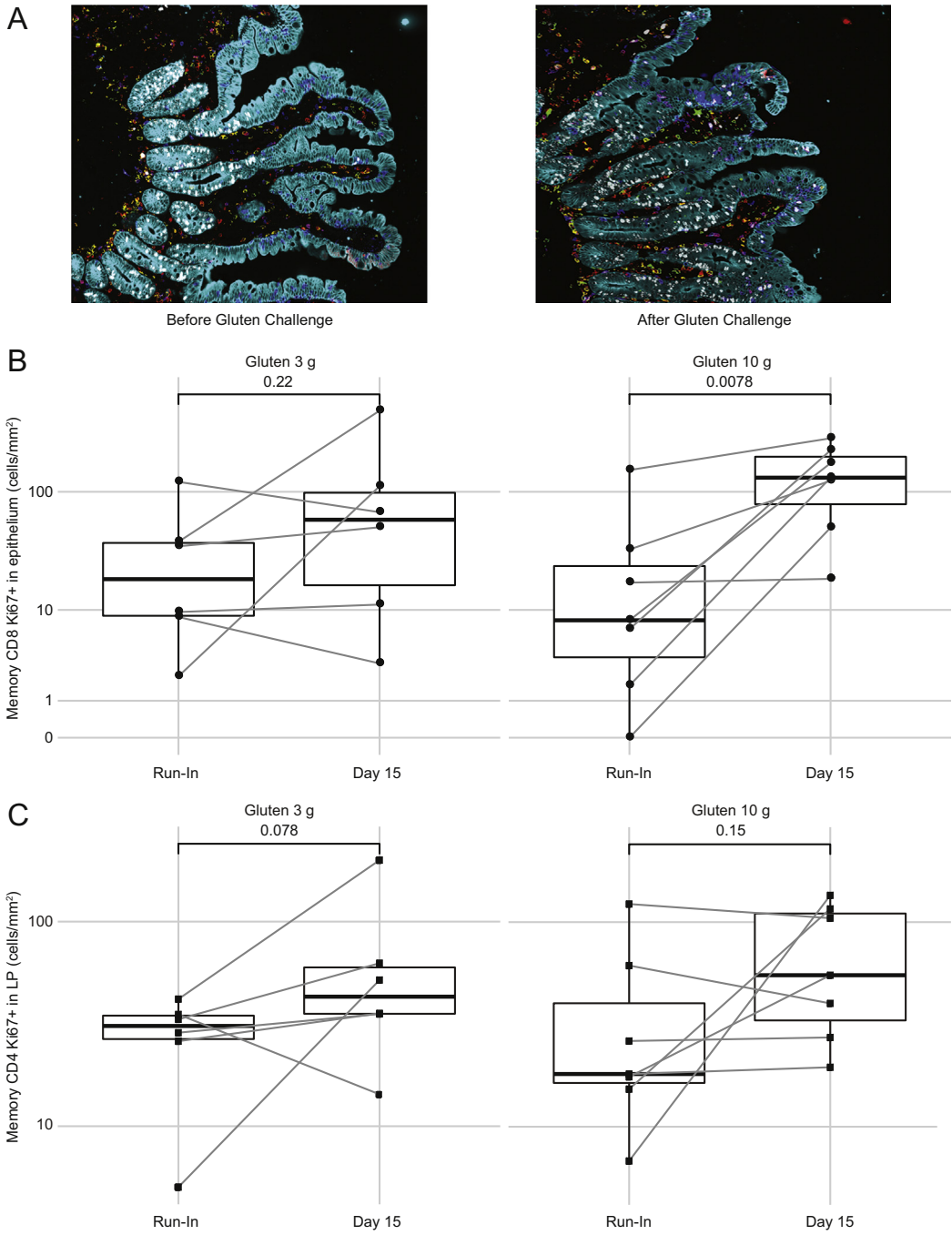
Supplementary Figure 1. Study design with timing of sample collection. Gluten was administered as dry powder mixed into warm liquid, either apple sauce or hot chocolate, and consumed in one sitting. No fasting before gluten challenge was required and there was no time limit or particular time of day for consumption specified. Gluten was prepared by the dietitian during clinic visits and by patients for home consumption. The primary end point was histology-based (Vh:Cd) and the secondary and exploratory end points were changes from baseline in blood gluten-specific T cells, specifically HLA-DQ-gluten tetramer staining of CD4 T cells and IFN-gamma–secreting T cells detected by ELISpot; gut-homing EM CD8 T cells detected by mass cytometry; plasma IL-2; urine GIP; CDSD symptoms; VCE enteropathy score; and correlation among outcome assessments. Samples for biomarkers were collected at the time points indicated by "X". Symptoms were collected daily from day –6 to the end of the study. [a]Quantitative histology was performed by author Dr Jeffrey Goldsmith at Harvard Medical School, Boston, MA. [b]Patients collected first morning void before daily gluten consumption. Urine was collected by patients and analyzed by the central laboratory. IF, immunofluorescence.



Supplementary Figure 2. Gastrointestinal symptoms using CDSD scores in patients receiving 3 g or 10 g of gluten. Data are median scores collected at run-in (days –7 to –1), GC (days 1–14), 7 days post-GC (days 15–21), and follow-up (days 22–42). Patient scores were calculated by addition of the individual responses for abdominal pain, nausea, diarrhea, bloating, and fatigue. GC, gluten challenge.



Supplementary Figure 3. Small intestinal mucosa pre (*A*) and post (*B*) post gluten challenge as captured by VCE.



Supplementary Figure 4. Intestinal T cells. Change in Ki67$^+$ memory CD8 and CD4 T cells. Representative images of formalin-fixed paraffin-embedded mucosal biopsy sections taken from the same patient before and after gluten challenge (*A*). Quantitation of Ki67$^+$ memory CD8 T cells in the epithelium (*B*). Quantitation of Ki67$^+$ memory CD4 T cells in the LP (*C*). Mucosal biopsies were stained with antibodies specific for CD45RO (*red*), CD4 (*green*), panCK (*cyan*), CD8 (*blue*), and Ki67 (*white*). The Ki67 (*white*) stained areas in the crypt present before and after gluten challenge are proliferating enterocytes. The Ki67$^+$ CD8 T cells are in the tip of the villi after gluten challenge. All stained images were registered, background autofluorescence removed, and subjected to tissue classification and cell segmentation. Cell-by-cell quantitative analysis provided the number of cells positive for each stain and marker coexpression data for phenotypic characterization. In panels *B* and *C*, the *lines* between data points connect data from individual patients taken at run in (day –6) and the day after 14-day gluten challenge was completed (day 15). Run-in and day 15 results were compared, and *P* values calculated based on 1-sided Wilcoxon signed rank test.

Supplementary Table 1. Antibodies for Multiplex Immunofluorescence

Target	Clone
CD3	F7.2.38
CD4	EPR6855, SP35
CD8	C8/144B
CD45RO	UCHL1
Ki67	SP6
PanCK	PCK_26 and AE1

NOTE. 5-μM sections were sequentially stained with antibodies directly conjugated with cyanine 3 or cyanine 5. After each staining round images were acquired, followed by dye inactivation. Images acquired before each new round of staining were used to remove autofluorescence.

Supplementary Table 2. Antibodies for Tetramer Staining

Reagent	Fluorescent label	Vendor
CD62L	BV605	BioLegend (San Diego, CA)
Integrin β7	BV711	BD Biosciences (San Diego, CA)
CD11c	APC	BD Biosciences
CD14 Alexa	Fluor 647	BioLegend
CD19	APC	BioLegend
CD56	APC	BioLegend
CD3	Alexa Fluor 700	BioLegend
CD4	APC/Cy7	BioLegend
CD45RA	BUV395	BD Biosciences
CD38	BUV737	BD Biosciences

NOTE. Peripheral blood mononuclear cells (PBMCs) were isolated $\leq$24 hours after the collection of blood from patients and frozen. CD4 T cells were isolated from cryopreserved PBMCs by magnetic bead-based selection (EasySep Human CD4 Positive Selection Kit II, StemCell Technologies, Vancouver, Canada) with all but 1 sample having $>$90% purity. AccuCell healthy donor $CD4^+$ T cells were spiked with primary human T cell clones specific for gliadin α-I and gliadin α-II and included as inter-assay controls. Three million CD4 T cells were stained with the HLA-DQ2 gluten-specific tetramers and antibodies to identify EM ($CD4^+/CD3^+/CD45RA^-/CD62L^-$), activated ($CD38^+$), gut-homing ($\beta7^+$) T cells and gate out other cell types (CD56, CD19, CD11c, and CD14). Controls for staining included unstained samples to detect autofluorescence and fluorescence-minus-1 samples to set compensation (includes all markers in panel except for 1) and for the tetramer (clinical and control samples). Flow cytometry was performed on a Fortessa 5-laser 18 color flow cytometer (BD Biosciences). Data were analyzed with Treestar FlowJo software, version 10 (Ashland, OR).
APC, allophycocyanin; BUV, Brilliant Ultraviolet; BV, Brilliant Violet; Cy, cyanine.

Supplementary Table 3. Antibodies for Mass Cytometry

Target	Clone	Mass label	Fluidigm catalog no.
CD8a	RPA-T8	^{141}Pr	Custom
CD19	HIB19	^{142}Nd	3142001B
CD38	HIT2	^{144}Nd	3144014B
CD4	RPA-T4	^{145}Nd	3145001B
CD3	UCHT1	^{154}Sm	3154003B
CD56 (NCAM)	B159	^{155}Gd	3155008B
CD197 (CCR7)	G043H7	^{159}Tb	3159003A
Integrin α4	9F10	^{156}Gd	Custom
Integrin β7	FIB504	^{163}Dy	Custom
CD45RA	HI100	^{170}Er	3170010B
CD45	HI30	^{89}Y	3089003B

NOTE. Cryopreserved peripheral blood mononuclear cells were thawed and washed in staining buffer (phosphate-buffered saline [PBS], 0.5% bovine serum albumin), blocked with Fc block (BD Biosciences), and stained with antibodies. Cells were stored in 3.2% (v/v) buffered paraformaldehyde in PBS containing iridium DNA intercalator (Fluidigm Corporation, San Francisco, CA) for $\leq$7 days. Cells were washed into water overnight before acquisition on a Helios mass cytometer (Fluidigm Corporation). Data acquisition was performed as described previously.[41] Data were analyzed and reduced with CellEngine software (Primitybio, Fremont, CA).

Supplementary Table 4. Demographic and Clinical Characteristics

Characteristic	Study cohort (n = 14)	Low gluten (3 g) (n = 7)	High gluten (10 g) (n = 7)	Low vs high gluten, *P* value
Demographic				
Sex, female, n (%)	11 (78.6)	6 (85.7)	5 (71.4)	1
Race, White, n *(%)*	14 (100.0)	7 (100.0)	7 (100.0)	1
Age, y, mean (SD)	43.7 (18.8)	41.4 (19.9)	46.0 (18.9)	.67
Years since diagnosis, mean (SD)	5.2 (4.7)	7.1 (6.1)	3.2 (1.5)	.13
Laboratory, n *(%)*				
HLA DQ2 homozygous	3 (21.4)	0 (0.0)	3 (42.9)	.42
HLA DQ2 heterozygous	5 (35.7)	3 (42.9)	2 (28.6)	
HLA DQB1*02 heterozygous	2 (14.3)	1 (14.3)	1 (14.3)	
HLA DQ8 heterozygous	3 (21.4)	2 (28.6)	1 (14.3)	
HLA DQ2 and DQ8	1 (7.1)	1 (14.3)	0 (0.0)	
Clinical, serologic, and histologic findings at study entry				
Vh:Cd, median (IQR)	2.1 (1.6–2.6)	2.0 (1.8–2.4)	2.5 (1.6–3.0)	.65
IEL, median (IQR)	26.1 (20.6–29.8)	23.2 (21.1–26.1)	26.7 (23.1–30.9)	.99
CDSD, overall mean (SD)	1.7 (2.1)	1.8 (2.8)	1.6 (1.4)	.92

NOTE. Continuous characteristics were compared with a 2-sample Student *t* test. Categorical variables were compared with Fisher exact test.
IQR, interquartile range.

Supplementary Table 5. Patient Serology

		Patients receiving 3 g gluten			Patients receiving 10 g gluten		
Visit	Test	Geometric, mean (SD)	Fold-change (SD)[a]	Significance[b] ($P < .05$)	Geometric, mean (SD)	Fold-change (SD)[a]	Significance[b] ($P < .05$)
Screening	TTG-IgA	3.0 (2.6)	—	—	7.6 (3.3)	—	—
Screening	DGP-IgG	2.8 (1.0)	—	—	3.6 (1.4)	—	—
Screening	DGP-IgA	5.8 (1.3)	—	—	6.2 (2.1)	—	—
Day 1	TTG-IgA	3.4 (2.3)	—	—	7.7 (3.4)	—	—
Day 1	DGP-IgG	2.8 (1.0)	—	—	3.3 (1.3)	—	—
Day 1	DGP-IgA	5.7 (1.3)	—	—	6.6 (1.8)	—	—
Day 15	TTG-IgA	3.6 (2.4)	1.1 (1.1)	.18	8.5 (3.4)	1.1 (1.3)	.42
Day 15	DGP-IgG	2.8 (1.0)	1.0 (1.0)	1	9.2 (3.2)	2.8 (3.0)	.059
Day 15	DGP-IgA	5.8 (1.3)	1.0 (1.0)	1	15.3 (2.2)	2.3 (2.0)	.058
Day 42	TTG-IgA	3.9 (2.6)	1.1 (1.2)	.11	14.0 (3.8)	1.8 (1.5)	.036
Day 42	DGP-IgG	2.8 (1.0)	1.0 (1.0)	1	17.9 (5.8)	5.4 (5.5)	.059
Day 42	DGP-IgA	5.7 (1.3)	1.0 (1.0)	1	17.3 (2.5)	2.6 (2.2)	.059

[a]Geometric mean values are presented for fold-change results.
[b]*P* values were calculated based on 2-sided Wilcoxon signed rank test.

Supplementary Table 6. Biomarker Correlation (Spearman Correlation)

Biomarker		Both doses				Low gluten (3 g)				High gluten (10 g)			
Assay 1	Assay 2	*r*	n	*P*	*Q*	*r*	n	*P*	*Q*	*r*	n	*P*	*Q*
CDSD	Vh:Cd	–0.35	14	.22	0.4	–0.21	7	.66	0.99	–0.071	7	.91	0.96
CDSD	IEL counts	–0.3	14	.3	0.47	–0.43	7	.35	0.84	0.11	7	.84	0.94
CDSD	VCE	–0.23	13	.46	0.59	–0.11	7	.84	1	0.14	6	.8	0.94
CDSD	Tetramer	–0.32	14	.26	0.42	0	7	1	1	–0.43	7	.35	0.82
CDSD	ELISpot	–0.19	14	.51	0.62	0	7	1	1	–0.5	7	.27	0.75
CDSD	IL-2	–0.28	14	.33	0.48	–0.64	7	.14	0.69	0.29	7	.56	0.83
CDSD	Gut-homing CD8 T cells	–0.69	12	.017	0.13	–0.31	6	.56	0.98	–0.71	6	.14	0.75
CDSD	Ki67$^+$ CD4 LP	0.022	13	.95	0.97	–0.37	6	.5	0.98	0.5	7	.27	0.75
CDSD	Ki67$^+$ CD8 IEL	–0.17	13	.58	0.67	–0.029	6	1	1	0.29	7	.56	0.83
Vh:Cd	IEL counts	0.54	14	.05	0.15	0.14	7	.78	1	0.39	7	.4	0.82
Vh:Cd	VCE	0.58	13	.043	0.15	0.071	7	.91	1	0.49	6	.36	0.82
Vh:Cd	Tetramer	0.55	14	.046	0.15	–0.071	7	.91	1	–0.11	7	.84	0.94
Vh:Cd	ELISpot	0.011	14	.98	0.98	–0.68	7	.11	0.69	–0.54	7	.24	0.75
Vh:Cd	IL-2	0.65	14	.015	0.13	0.071	7	.91	1	0.39	7	.4	0.82
Vh:Cd	Gut-homing CD8 T cells	0.72	12	.011	0.13	0.49	6	.36	0.84	0.43	6	.42	0.82
Vh:Cd	Ki67$^+$ CD4 LP	0.038	13	.91	0.97	0.14	6	.8	1	0.14	7	.78	0.94
Vh:Cd	Ki67$^+$ CD8 IEL	0.61	13	.03	0.13	0.26	6	.66	0.99	0.86	7	.024	0.75
IEL counts	VCE	0.64	13	.021	0.13	–0.11	7	.84	1	0.77	6	.1	0.75
IEL counts	Tetramer	0.64	14	.016	0.13	0.5	7	.27	0.84	0.29	7	.56	0.83
IEL counts	ELISpot	0.27	14	.34	0.48	0	7	1	1	0.18	7	.71	0.92
IEL counts	IL–2	0.83	14	.0004	0.018	0.93	7	.0067	0.3	0.71	7	.088	0.75
IEL counts	Gut-homing CD8 T cells	0.5	12	.1	0.25	0.14	6	.8	1	0.26	6	.66	0.92
IEL counts	Ki67$^+$ CD4 LP	0.68	13	.014	0.13	0.89	6	.033	0.69	0.75	7	.066	0.75
IEL counts	Ki67$^+$ CD8 IEL	0.61	13	.03	0.13	0.77	6	.1	0.69	0.61	7	.17	0.75
VCE	Tetramer	0.34	13	.26	0.42	–0.64	7	.14	0.69	–0.26	6	.66	0.92
VCE	ELISpot	0.4	13	.18	0.37	0.64	7	.14	0.69	–0.37	6	.5	0.83
VCE	IL-2	0.38	13	.2	0.37	–0.14	7	.78	1	0.2	6	.71	0.92
VCE	Gut-homing CD8 T cells	0.45	11	.17	0.37	–0.31	6	.56	0.98	–0.1	5	.95	0.97
VCE	Ki67$^+$ CD4 LP	0.15	12	.64	0.71	–0.31	6	.56	0.98	0.71	6	.14	0.75
VCE	Ki67$^+$ CD8 IEL	0.2	12	.53	0.63	–0.43	6	.42	0.9	0.77	6	.1	0.75
Tetramer	ELISpot	0.37	14	.19	0.37	–0.25	7	.59	0.99	0.68	7	.11	0.75
Tetramer	IL-2	0.58	14	.032	0.13	0.5	7	.27	0.84	0	7	1	1
Tetramer	Gut-homing CD8 T cells	0.45	12	.15	0.33	–0.26	6	.66	0.99	0.37	6	.5	0.83
Tetramer	Ki67$^+$ CD4 LP	0.033	13	.92	0.97	0.49	6	.36	0.84	–0.29	7	.56	0.83
Tetramer	Ki67$^+$ CD8 IEL	0.29	13	.34	0.48	0.6	6	.24	0.84	–0.29	7	.56	0.83

Supplementary Table 6. Continued

Biomarker		Both doses				Low gluten (3 g)				High gluten (10 g)			
Assay 1	Assay 2	*r*	n	*P*	*Q*	*r*	n	*P*	*Q*	*r*	n	*P*	*Q*
ELISpot	IL-2	0.2	14	.49	0.62	0	7	1	1	0.071	7	.91	0.96
ELISpot	Gut-homing CD8 T cells	0.26	12	.42	0.55	–0.6	6	.24	0.84	0.43	6	.42	0.82
ELISpot	Ki67$^+$ CD4 LP	–0.27	13	.37	0.51	–0.31	6	.56	0.98	–0.18	7	.71	0.92
ELISpot	Ki67$^+$ CD8 IEL	–0.34	13	.25	0.42	–0.43	6	.42	0.9	–0.61	7	.17	0.75
IL-2	Gut-homing CD8 T cells	0.51	12	.094	0.25	0.029	6	1	1	0.6	6	.24	0.75
IL-2	Ki67$^+$ CD4 LP	0.53	13	.064	0.18	0.83	6	.058	0.69	0.54	7	.24	0.75
IL-2	Ki67$^+$ CD8 IEL	0.62	13	.029	0.13	0.49	6	.36	0.84	0.46	7	.3	0.8
Gut-homing CD8 T cells	Ki67$^+$ CD4 LP	0.036	11	.92	0.97	0.6	5	.35	0.84	–0.14	6	.8	0.94
Gut-homing CD8 T cells	Ki67$^+$ CD8 IEL	0.53	11	.1	0.25	0.7	5	.23	0.84	0.086	6	.92	0.96
Ki67$^+$ CD4 LP	Ki67$^+$ CD8 IEL	0.59	13	.038	0.14	0.77	6	.1	0.69	0.57	7	.2	0.75

NOTE. Correlations of maximal change between of pairs of biomarkers is presented with the accompanying number of samples used for the correlation, *P* values, and *Q* values (false discovery rate). Vh:Cd change was inverted because decreasing Vh:Cd signifies increasing severity. Analyses of correlations among CDSD, Vh:Cd, IEL, VCE, ELISpot, and tetramer staining were prespecified, whereas analyses of correlations between IL-2 and IF were exploratory.