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HLA-DQ:gluten tetramer test in blood gives better detection of coeliac patients than biopsy after 14-day gluten challenge

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ABSTRACT

Objective: Initiation of a gluten-free diet without proper diagnostic work-up of coeliac disease is a frequent and demanding problem. Recent diagnostic guidelines suggest a gluten challenge of at least 14 days followed by duodenal biopsy in such patients. The rate of false negative outcome of this approach remains unclear. We studied responses to 14-day gluten challenge in subjects with treated coeliac disease.

Design: We challenged 20 subjects with biopsy-verified coeliac disease, all in confirmed mucosal remission, for 14 days with 5.7 grams per oral gluten daily. Duodenal biopsies were collected. Blood was analysed by multiplex assay for cytokine detection, and by flow cytometry using HLA-DQ:gluten tetramers.

Results: Nineteen participants completed the challenge. Villous blunting appeared at end of challenge in five of 19 subjects. Villous height to crypt depth ratio reduced with at least 0.4 concomitantly with an increase in intraepithelial lymphocyte count of at least 50% in nine of 19 subjects. IL-8 plasma concentration increased by more than 100% after four hours in seven of 19 subjects. Frequency of blood $CD4^+$ effector-memory gut-homing HLA-DQ:gluten tetramer-binding T cells increased by more than 100% on day 6 in 12 of 15 evaluated participants.

Conclusion: A 14-day gluten challenge was not enough to establish significant mucosal architectural changes in majority of coeliac disease patients (sensitivity ≈ 25 – 50%). Increase in $CD4^+$ effector-memory gut-homing HLA-DQ:gluten tetramer-binding T cells in blood six days after gluten challenge is a more sensitive and less invasive biomarker that should be validated in a larger study.

SIGNIFICANCE OF THIS STUDY:

1. What is already known about this subject?

- Many subjects maintain a gluten-free diet without initial work-up for coeliac disease.
- For subjects in this situation a recommended work-up for coeliac disease requires a gluten challenge for two to eight weeks, followed by a duodenal biopsy. This procedure may cause unacceptable symptoms in some patients.
- The recommendation of a two-week gluten challenge is based on limited evidence, and the sensitivity of this procedure is not well validated.

2. What are the new findings?

- A two-week gluten challenge is not enough to detect coeliac disease by conventional histological evaluation of duodenal biopsies.
- The sensitivity of histological evaluation can be increased by applying morphometry in a paired set of duodenal biopsies taken before and after gluten challenge.
- Following the first dose of gluten there was a two-fold change in plasma concentration of IL-8 and MIP-1β in some of the subjects with coeliac disease in remission.
- A two-fold change in gluten-specific T-cell response in blood, measured by HLA-DQ:gluten tetramers, was detected after six days of gluten challenge in a majority of subjects with coeliac disease in remission.

3. How might it impact on clinical practice in the foreseeable future?

- This study lowers expectations of a positive duodenal histology after a two-week gluten challenge and supports clinical decision-making in favour of longer duration of gluten challenge.
- A paired set of duodenal biopsies to achieve a higher diagnostic sensitivity should be considered if the patient may have difficulties in completing the recommended duration of gluten challenge.
- A flow-cytometric assay for gluten-specific T cells in blood, using HLA-DQ:gluten tetramers, can be applied to detect coeliac disease after a short gluten challenge.

INTRODUCTION

Coeliac disease is a gluten dependent disorder characterized by changes in gut mucosal architecture and presence of autoantibodies to transglutaminase 2 (TG2) and antibodies to deamidated gliadin peptides (DGP).[1, 2] The disease pathology is controlled by gluten-specific $CD4^+$ T cells that recognize deamidated gluten peptides presented by the disease associated HLA molecules DQ2.5, DQ2.2 or DQ8.[3, 4] Elevated serum levels of anti-TG2 IgA and anti-DGP IgG are sensitive and specific markers for detection of coeliac disease.[5] Finding increased numbers of intraepithelial lymphocytes (IEL), hypertrophic crypts and partial or complete blunting of villi in duodenal biopsies is considered the gold standard for establishment of the diagnosis.[1]

The only available treatment of coeliac disease is a strict and life-long exclusion of gluten from the diet. Popular awareness of potential gluten-related health problems has led to increasing number of individuals pursuing self-prescribed gluten-free diet, without an adequate diagnostic work-up of coeliac disease.[6] This practice poses a diagnostic challenge to clinicians, as sensitivity of available tests for diagnosis of coeliac disease reduces significantly in subjects who are not eating gluten. In such cases, recent guidelines recommend challenge with 3 g gluten daily for at least two weeks, prolonged to eight weeks if possible, followed by duodenal biopsy.[1, 7, 8] The recommended duration of minimum two weeks of gluten challenge is based on a single study and has not been extensively validated.[8]

Consumption of gluten may elicit unacceptable symptoms in patients undergoing the challenge and failure to complete the protocol. This may lead to frustration for patients and a failed diagnostic work-up in clinical settings, but also to a significant dropout rate in context of therapeutic studies requiring gluten challenge.[9-12] Striving for shorter duration of gluten challenge warrants response parameters that are more sensitive than the commonly used modified Marsh classification (Marsh type).[13, 14] Continuous measures, such as villus height

to crypt depth (Vh/Cd) ratio and intraepithelial lymphocyte (IEL) counts have therefore been suggested and validated.[15] Additionally, detection of gluten-specific T cells in blood after a short gluten challenge has been proposed as a sensitive test for coeliac disease, either performed as an enzyme-linked immunospot assay after incubation of blood cells with gluten,[16, 17] or direct detection of gluten-specific T cells in blood cells with the use of HLA-DQ:gluten tetramers and flow cytometry.[18-20] Other potential parameters for prediction of disease specific inflammation may include cytokine production in the early phases of a gluten challenge.[21]

In this study we examined whether a 14-day gluten challenge was enough to invoke villous blunting in well-treated subjects with coeliac disease. We also asked whether the sensitivity of a short gluten challenge can be improved by applying methods accessible in clinical practice; such as measurement of Vh/Cd ratio and IEL-counting, in addition to novel methods; such as detection of gluten-specific T cells in blood on day 6 and detection of cytokines in blood within few hours after gluten challenge.

METHODS:

Inclusion and recruitment

All participants had biopsy confirmed coeliac disease and were in remission on a gluten-free diet at the time of inclusion. Remission was evaluated by a routine duodenal biopsy and defined by Marsh type 0 or 1 and negative anti-TG2 IgA level. For a complete list of inclusion and exclusion criteria, see Supplementary table 1. For further details on the participant recruitment, see Supplemental methods.

Gluten challenge protocol

A baseline duodenal biopsy was taken, in most cases one to two weeks before the onset of challenge, to confirm remission in all participants (Supplemental figure 1). The participants ingested a 50 g muesli bar daily for 14 days, containing 7.6 g of gluten flour (5.7 g gluten

protein), free of fermentable oligo-, di-, monosaccharides and polyols (Supplementary table 2). The muesli bars were developed and produced by Monash University, Melbourne. The content of gluten in the muesli bars was confirmed by ELISA and mass-spectrometry (nano-LC-MS/MS) (data not shown). Apart from the gluten-containing muesli bar, the participants continued their regular gluten-free diet. The participants underwent the first day of gluten challenge under medical supervision.

Duodenal histopathology

Gastroduodenoscopy was done at baseline and on day 14 of gluten challenge. At both time points four biopsies were collected from the second part of duodenum. The biopsies were subjected to an initial non-blinded assessment of Marsh type, and then de-identified for a blinded evaluation of Marsh type, Vh/Cd and IEL-count. See Supplemental methods for details. Vh/Cd > 2 was considered normal.[10, 22] An IEL-count of 25 was used as cut-off between Marsh type 0 and 1.[22]

Antibody tests, HLA-typing and cytokine analysis

Measurements of anti-TG2 IgA (normal range < 3 units/ml, VarElisa Celikey IgA, Phadia, Freiburg, Germany) and anti-DGP IgG (normal range < 20 units, QUANTA Lite™ Gliadin IgG II, INOVA Diagnostics Inc., San Diego, CA) were done in serum at baseline and then on day 6, day 14 and day 28 after start of challenge. Total IgA was only measured in cases with anti-DGP IgG elevation without anti-TG2 IgA elevation. All included participants were typed for HLA-DQA1 and HLA-DQB1 alleles (full genomic HLA-typing, LABType™ SSO, ONE LAMBDA, Los Angeles, CA).

At the first day of challenge, plasma samples for cytokine determination were collected prior to gluten challenge, and then two, four and six hours after challenge. Samples were kept frozen at -80°C and later analysed with a 27-plex bead assay (Bio-Plex Pro™ Human Cytokine 27-plex Assay, Bio-Rad, Hercules, CA). Data analysis was done with Bio-Plex MAGPIX™ Multiplex Reader and Bio-Plex Manager 6.1 software (Bio-Rad, Hercules, CA).

Frequency estimation of gluten-specific T cells using HLA-DQ:gluten tetramers

We analysed gluten-specific T cells at baseline and on day 6 of gluten challenge with HLA-DQ:gluten tetramers by flow cytometry as described elsewhere.[18-20] Recombinant and biotinylated HLA-DQ2.5 molecules[23] and HLA-DQ8 molecules[24] with sequences representing peptide epitopes tethered to DQβ-chain were used for generation of HLA-DQ tetramers by multimerization on fluorophore-conjugated streptavidin. The DQ2.5:glia-α1a, DQ2.5:glia-α2, DQ2.5:glia-ω1 and DQ2.5:glia-ω2, and DQ8:glia-α1 and DQ8:glia-γ1b epitopes were displayed in context of HLA-DQ2.5 and HLA-DQ8 molecules, respectively. For further details, see Supplemental methods. The cells were analysed by flow cytometry and gated for $CD4^+CD3^+CD11c^-CD14^-CD19^-CD56^-CD45RA^-CD62L^-integrin\beta7^+$HLA-DQ:gluten-$tetramer^+$ (HLA-DQ:gluten-$tetramer^+\beta7+T_{EM}$) (Supplemental figure 2). The number of HLA-DQ:gluten-$tetramer^+\beta7+T_{EM}$ was normalized to 10^6 $CD4^+$ cells in the sample for frequency estimation.

Patient reported outcomes

Symptoms were scored by the Celiac Symptom Index (CSI),[25] visual analogue scales (VAS) and the Gastrointestinal Symptoms Rating Scale Irritable Bowel Syndrome version (results not shown).[26] See Supplemental methods for further details.

Statistics

Statistical analysis was done on GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA) and SPSS (IBM SPSS Statistics V22.0, North Castle, NY). Power transformations were applied where necessary. See Supplemental methods for further details.

RESULTS

Participant characteristics and completion of challenge

Twenty participants were included, of whom 16 were women and four were men, with mean age 41.6 years (SD 16.5) and mean BMI 23.8 kg/m^2 (SD 3.9) (Supplementary table 3). The

median duration of gluten-free diet was 139 months, ranging from 26 to 473 months. Seventeen participants were HLA- DQ2.5 and the remaining three were HLA-DQ8.

Nineteen participants completed the gluten challenge and underwent both gastro-duodenoscopies. One participant, who did not complete the gluten challenge, had a flare of previously known atopic dermatitis from the second day of challenge. The challenge was stopped after three days and she was prescribed a high dose steroid therapy with effect on resolution of symptoms.

Only a small proportion of subjects had villous blunting after 14 days of gluten challenge

During the initial, non-blinded evaluation, prior to gluten challenge, all duodenal biopsies were reported as either Marsh type 0 or 1. However, during the blinded assessment, one participant was considered to have Marsh type 3 at baseline biopsy, whereas the remaining baseline Marsh types were 0 or 1 (Figure 1A). Finally, blinded day 14 histology results showed Marsh type 3 in five biopsies (Table 1).

ID	HLA-DQ:gluten tetramer-test fold change day 6	IEL fold change day 14	Vh/Cd difference day 14	IL-8 fold change 4 hours	MIP-1β fold change 4 hours	Vh/Cd Day 14	Marsh type day 14	Anti-TG2 IgA U/ml day 28	Anti-DGP IgG U day 28
	Cut-off = 2	*Cut-off = 1.5*	*Cut-off = 0.4*	*Cut-off = 2*	*Cut-off = 2*	*Cut-off = 2*	*Cut-off = 3*	*Cut-off = 3*	*Cut-off = 20*
CD1343	2.32	4.09	1.27	2.58	2.03	1.52	3	<1	6.0
CD442	5.57	2.12	-0.01	6.21	6.61	1.87	3	3.9	10.0
CD1295	ND	2.03	1.08	6.12	3.42	1.11	3	<1	<5
CD1300	72.84	3.34	0.78	2.05	1.87	1.43	3	<1	<5
CD1302	2.93	1.77	0.40	2.16	2.08	2.06	1	<1	<5
CD1351	ND	1.60	0.59	2.56	2.70	2.40	1	1.4	6.0
CD1378	ND	ND	ND	3.94	3.82	ND	ND	ND	ND
CD1296	17.29	2.22	1.14	1.23	0.92	1.63	3	<1	<5
CD1340	77.00	2.78	0.35	1.25	1.38	2.17	1	3.5	86.0
CD1353	7.57	1.74	0.57	1.27	1.28	2.16	1	<1	<5
CD1379	4.74	1.54	1.16	ND	ND	2.53	1	<1	<5
CD1342	13.13	4.48	0.85	1.25	1.03	2.01	1	1.9	<5
CD1339	6.94	1.63	0.13	1.46	1.65	1.84	1	<1	6.0
CD1303	2.61	0.86	0.18	1.38	1.11	2.59	1	<1	44.0

CD1299	11.18	1.11	-0.21	1.92	1.00	3.44	1	<1	<5
CD1298	1.07	1.07	0.52	0.66	1.00	2.75	0	<1	<5
CD1178	0.61	1.36	0.36	0.81	1.09	2.52	1	<1	10.0
CD1284	ND	0.99	0.08	1.07	1.07	2.70	1	<1	<5
CD1366	1.00	0.69	-0.28	1.18	0.77	3.19	0	<1	<5
CD1294	ND	1.04	-0.60	1.24	0.97	3.07	0	<1	<5

Table 1: Response parameters in 14-day gluten challenge.

Note. HLA-DQ:gluten tetramer-test, HLA-DQ:gluten-tetramer$^{+}\beta7{+}T_{EM}$ / 10^6 CD4+ cells; *ND, not done*; U, units. *Response parameters in the top row are sorted by decreasing sensitivity of response, showing HLA-DQ:gluten tetramer fold change to be the most, and antibody level to be the least sensitive parameters for coeliac disease. The second row shows the cut-off values used for each parameter. Positive responses are marked in grey. The subjects in the first column are sorted by the number of positive responses (with discretion applied to missing values). Fold change in each parameter is calculated by dividing the level at the annotated time point by baseline level. Vh/Cd difference day 14 is calculated by subtracting day 14 level from baseline level.*

The average Vh/Cd changed significantly from 2.70 at baseline to 2.26 on day 14 of gluten challenge (p=0.002) (Figure 1B). Seven of 19 subjects had biopsy Vh/Cd < 2.0 on day 14, but two had biopsy Vh/Cd < 2 already at baseline.[10, 27] Using cut-off for significant absolute change in Vh/Cd ≤ 0.4 as proposed by others,[15] we found significant decrease from baseline to day 14 in 10 of 19 participants (Table 1).

IEL-count is more sensitive than mucosal architectural changes

The mean IEL count increased significantly from 23.5 at baseline to 40.9 on day 14 of gluten challenge (p<0.001) (Figure 1C). By applying a significance cut-off of 50% increase in IEL-count from baseline, based on investigations done in haematoxylin and eosin stained biopsies by others,[15] we were able to detect response in 12 of 19 participants (Table1). Nine

of these 12 participants who responded with significant IEL-change, did also have significant Vh/Cd absolute reduction of 0.4.

Antibody levels remained low 28 days after start of gluten challenge

Anti-TG2 IgA levels were negative at baseline for all participants in accordance with the inclusion criteria (Figure 2A) and rose to elevated levels in two participants by day 28 (Table 1). Similarly, two participants were positive for anti-DGP IgG at day 28 (Figure 2B and Table 1) of whom one was contemporaneously anti-TG2 IgA-positive.

Significantly elevated concentration for several cytokines a few hours after gluten challenge

Thirteen of 27 tested cytokines showed significant increase in plasma concentration on either four or six hours after gluten challenge compared to baseline (Figure 2C). Three cytokines had a highly significant increase ($p<0.001$); IL-8, IP-10 and Eotaxin and peak median fold changes of 1.6, 1.6 and 1.4 respectively, all peaking at six hours (Supplementary table 3). Some of the cytokine concentrations were found to be below the lower detection limit (LDL) for almost all subjects; IL-2 (LDL = 0.28 pg/ml), IL-6 (LDL = 0.44 pg/ml), IL-15 (LDL = 4.08 pg/ml), GM-CSF (LDL = 1.2 pg/ml), MCP-1 (LDL = 5.04 pg/ml) and VEGF (LDL = 9.36 pg/ml). Other measured cytokines did not show any significant change from baseline levels; IL-10, IL-13, FGF basic, PDGF-bb, INF γ, G-CSF, TNF α and RANTES.

Increased levels of gluten-specific T cells in blood were measured in the majority of participants on day 6

An arbitrary cut-off of twofold change was defined for the frequency of HLA-DQ:gluten-tetramer$^{+}\beta7^{+}T_{EM}$ / 10^{6} $CD4^{+}$ cells (day 6 level divided by baseline level), and 12 of 15 participants were found to respond accordingly (Table 1). Flow cytometry data at day 6 were not available for four participants due to technical reasons, and excluded for one participant due to immune suppressive treatment. The median numbers of HLA-DQ:gluten-

tetramer$^{+}\beta7^{+}T_{EM}$ / 10^{6} $CD4^{+}$ cells increased significantly ($p<0.001$) from 4.2 at baseline to 22.9 on day 6 (Figure 3A). Surprisingly, one non-responder had no detectable HLA-DQ:gluten-tetramer$^{+}\beta7^{+}T_{EM}$ at baseline nor on day 6 (CD1366) (Table 1). This participant was diagnosed in early childhood in the 1970s, and had kept a strict gluten-free diet since.

We looked for CD38 expression in the last half of the study, thus obtaining data from 10 participants for this marker (Figure 3B). The median CD38 expression in HLA-DQ:gluten-tetramer$^{+}\beta7^{+}T_{EM}$ was 1.8% (range 0 – 30.2%) at baseline, and increased significantly ($p<0.001$) on day 6 with median 91.3% (range 79.9% – 99.5%). In contrast, HLA-DQ:gluten tetramer-negative control-cells of similar phenotype (integrin-$\beta7^{+}T_{EM}$) did not display any signficiant difference ($p=0.085$) from baseline to day 6.

Increased symptoms during the first week of gluten challenge, but unaltered quality of life scores

Gastrointestinal symptoms, as scored by the CSI, increased significantly ($p=0.002$) from baseline to the end of challenge from a median score of 24 (interquartile range 7) to 27 (interquartile range 8) on a 16 – 80 scale (Figure 4A). VAS-scores showed significant changes in stool consistency from baseline to week 1 ($p=0.046$), and in flatulence from baseline to week 2 ($p=0.019$) (Supplemental figure 3). VAS scores rating overall symptoms on day 1 of gluten challenge showed a non-significant trend ($p=0.060$) towards a higher symptom load at 6 hours post challenge, compared to baseline (Figure 4B).

Significant correlation between symptom response and change in concentration for IL-8 and MIP-1β on day 1 of gluten challenge

We calculated fold change in cytokine concentrations (given time point / baseline) for significantly increased cytokine concentrations, and analysed for correlations to fold change in overall symptoms at day 1 of gluten challenge (Supplementary table 4). A significant correlation was found at 4 hours for IL-8 ($p=0.015$) and MIP-1β ($p=0.015$). As a measure of

the gluten-induced response of these cytokines, we could find a twofold change in concentration, chosen as an arbitrary cut-off, in seven of 19 participants for IL-8 and in six of 19 for MIP-1β (Table 1).

Correlation between outcome parameters and baseline parameters

Fold change in level of blood HLA-DQ:gluten-tetramer^{+}β7^{+}T$_{EM}$ (day 6 / baseline) showed good correlation (r_s=0.62) with fold change in IEL-count (day 14 / baseline), but the correlation was not significant (p=0.13) after correction for multiple comparisons (Supplementary table 5). Fold change in IEL-count was significantly correlated to fold change in Vh/Cd (day 14 / baseline) (p=0.010). The baseline IEL-count was negatively correlated to fold change in IEL-count (p=0.010) and near-significant negatively correlated to fold change in Vh/Cd (p=0.064). Baseline levels of HLA-DQ:gluten-tetramer^{+}β7^{+}T$_{EM}$ had a significant correlation to fold change of IL-8 (4 hours / baseline) (p=0.007) and MIP-1β (4 hours / baseline) (p=0.003), and to fold change in day 1 VAS overall symptoms (peak / baseline) (p=0.045). Fold change for CSI (day 14 / baseline), antibody levels (day 28 / baseline) or baseline Vh/Cd were neither found to be significantly correlated to each other, nor to any other tested variable.

DISCUSSION

In this study we investigated several different aspects of the response to a 14-day gluten challenge and asked whether 14 days are enough to elicit mucosal architectural changes. Among 19 adults with coeliac disease in remission, we found that the 14-day gluten challenge, performed in accordance with the recommendations for minimum duration in recent guidelines,[1, 7] resulted in detectable villous blunting (Marsh type 3) in only five subjects, whereas the remaining 14 were test negative. A 14-day gluten challenge should therefore be considered insufficient for detection of coeliac disease.

A previous study by Leffler et al.,[8] on which recent recommendations of 14-day gluten challenge were based, reported Marsh type 3 in biopsies from 13 of 19 participants at the end of a 14-day gluten challenge, which is a significantly higher proportion than in our study. Although the authors did not state the rate of villous blunting at baseline, it appears likely that several participants already had mucosal architectural changes at baseline, as 8 of 19 participants had Vh/Cd ≤ 2, and mean Vh/Cd was 2.21 at baseline, compared to a mean Vh/Cd of 2.70 at baseline in our study. This difference may partly reflect differences in baseline treatment status, as their study cohort had a shorter duration on gluten-free diet prior to gluten challenge (average 65 versus median 139 months). The content of gluten was confirmed in both studies and there is no reason to assume that the two formulations (muesli bars versus bread) or sources of gluten were qualitatively different. The dose of gluten in Leffler et al. was 3 g and 7.5 g gluten daily in equal sized groups (with no response difference between groups), in contrast to 5.7 g gluten daily in our study. Measurement of outcome (Marsh type and morphometry) was done with the same technique in both studies and should not be a sufficient explanation for difference in outcome. Therefore we believe the differences in baseline treatment and remission status to be the main explanation for the observed differences in endpoint histopathology between our study and Leffler et al.

The optimal dose of gluten in a short challenge is not known and should probably be seen in conjunction with the duration of the challenge. One study of 6-week gluten challenge in adults in mucosal remission used daily doses of 1.5, 2, 3 and 6 g gluten, showing a clear dose response effect, diminishing towards the higher doses, as doses of 3 g and 6 g were both able to give Vh/Cd ≤ 2 in about 70% of the subjects.[10] It is, however, not clear when the villous blunting occurred during the 6-week time frame. Thus, although a daily gluten dose of 3 g may be sufficient for a 6-week challenge, it may not be sufficient for a 14-day challenge, as seen in our study where the use of 5.7 g gluten daily only gave Vh/Cd ≤ 2 in approximately one third of the participants.

An alternative strategy for response evaluation could be repeated sets of duodenal biopsies, before and after gluten challenge. This approach could provide a more sensitive readout than the recommended practice of only taking one set of biopsies at the end of a gluten challenge. Two parameters, i.e. an absolute change in Vh/Cd of 0.4 and an IEL change of about 50% in haematoxylin and eosin stained biopsies, have previously been validated in this context.[15] Although we were able to double the sensitivity of the 14-day gluten challenge by applying these two cut-offs for response evaluation, we still found the sensitivity to be unsatisfactory, at around 50%.

The kinetics of coeliac disease specific antibodies have been shown to be quite slow in the context of gluten challenge; 3-day, 6-week and 12-week challenges with different doses of gluten gave seroconversion of anti-TG2 IgA-levels in 0%, 30% and 43%, respectively.[9, 11, 28] Our findings are in accordance with these observations, showing 10% anti-TG2 IgA seroconversion rate after 14-day gluten challenge, in contrast to 55% in Leffler et al. The inclusion of subjects that were only partially in remission in the Leffler et al. study, in addition to differences in cut-offs and dynamic range between the different assays, could potentially explain the different degrees of seroconversion in their compared to our study.

We found 13 cytokines with significantly increased concentrations in blood on day 1 of gluten challenge, but the measured responses were too weak for most of the cytokines to represent potential candidates as clinical outcome parameters in gluten challenge. The increase in IL-8 and MIP-1β at 4 hours after gluten challenge was particularly notable with regard to significance level, and significant correlations to symptom response and baseline numbers of blood HLA-DQ:gluten-tetramer^{+}β7^{+}T_{EM}. IL-8 and MIP-1β are known to be pro-inflammatory chemokines related to innate immune responses,[29-32] but also to adaptive responses.[33] IL-8 in particular, has been shown to be specific to gluten exposure in coeliac disease.[21, 34-36] A recent therapeutic study showed a symptom-associated elevation of IL-8 and MIP-1β (along with IL-2, IL-10, GM-CSF, TNF-α and MIP-1a) in blood, 4 hours after intra-dermal injection of immunodominant gluten peptides.[37] Thus, although IL-8 and MIP-1β lacked sensitivity as

biomarkers for coeliac disease, their association to symptom response and HLA-DQ:gluten-tetramer$^{+}\beta7^{+}T_{EM}$ / 10^6 $CD4^{+}$ cell levels in blood may point to a role of the adaptive immune system through circulatory, or even tissue-resident, gluten-specific cells in causing symptoms in the early phases of gluten-induced inflammation in coeliac disease.[38, 39]

The use of tetramers, which are fluorescence-emitting complexes of peptide-antigens tethered to HLA-molecules, has allowed us to identify the T cells specific to a particular peptide-antigen of interest. We used this technology to identify gluten-specific T-cells known to be central in the pathogenesis of coeliac disease and detected at least 100% increase in numbers of HLA-DQ:gluten-tetramer$^{+}\beta7^{+}T_{EM}$ / 10^6 $CD4^{+}$ T-cells in 12 of 15 participants. This result is in line with a previous gluten challenge study that additionally found the HLA-DQ:gluten tetramer test (in a different version than in the current study) to correlate well with the enzyme-linked immunospot assay for detection of gluten-specific T-cells.[19] The HLA-DQ:gluten tetramer test has since improved by applying a bead-based enrichment protocol and supplementary cell surface markers,[20] and was therefore preferred to the enzyme-linked immunospot assay test in the current study. Moreover, we found that the expression of the activation marker CD38 by HLA-DQ:gluten-tetramer$^{+}\beta7^{+}T_{EM}$ increased from maximum 30% in subjects on a strict gluten-free diet, to minimum 80 % on day 6 of gluten challenge. Thus, we not only confirm the observations of a previous study, where CD38 was shown to be expressed on the majority of HLA-DQ:gluten tetramer-binding cells after a gluten challenge,[40] but we also for the first time demonstrate the rapid increase from baseline levels. Our results clearly demonstrate that the gluten-specific T-cell response in blood on day 6 is a sensitive and fast reacting parameter for gluten exposure in coeliac disease. Based on previous results, a three-day challenge, and not continuous challenge in six days as was done in the current protocol, should suffice.[17-19] If undertaking a gluten challenge as part of the work-up, this three-day challenge monitored by a near to non-invasive HLA-DQ:gluten tetramer test should represent an attractive option for patients and clinicians alike.

Our results raise the possibility that a gluten challenge is not needed to establish the diagnosis of coeliac disease in subjects who are on a gluten-free diet. Increased level of HLA-DQ:gluten-tetramer$^+\beta7^+T_{EM}$ appears to be a marker of coeliac disease regardless of dietary regime as all, except one, of our participants with biopsy proven coeliac disease had detectable levels at baseline. Also in a previous study using a similar, but not identical protocol (with fewer HLA-DQ:gluten tetramers and without the gut-homing marker integrin β7), eleven of 13 HLA-DQ2.5$^+$ treated coeliac disease patients had HLA-DQ:gluten-tetramer$^+T_{EM}$ / 10^6 CD4$^+$ cells ≥ 1, while all ten control subjects were below this cut-off.[20] A study designed to assess this diagnostic approach (clinicaltrials.gov identifier: NCT02442219) should provide further insight in this regard. An increasing number of encouraging results may propel initiatives for commercialization and introduction of this test for clinical use in the foreseeable future by overcoming the limitation of current small-scale production of HLA-DQ:gluten tetramers for academic research purposes only.

The large subject variability in the range of increase of HLA-DQ:gluten-tetramer$^+\beta7^+T_{EM}$ upon gluten challenge is striking. The reason for this large variation in response is currently unknown, but a non-significant trend towards a correlation to baseline IEL-counts may suggest that this can be related to degree of mucosal inflammation status prior to gluten challenge. In addition, we observed lower than median baseline levels of HLA-DQ:gluten-tetramer$^+\beta7^+T_{EM}$ in the three non-responders that showed less than twofold change on day 6, further suggesting an association between the size of gluten-specific memory T-cell population and the degree of response to antigen stimulus in the form of gluten challenge.

Patient reported outcomes are gaining increasing importance, not least for monitoring efficacy of drug intervention in coeliac disease.[41] Although we saw a significant increase in gastrointestinal symptoms during the 14-day gluten challenge, this symptom response did not correlate to changes in other objective outcome measures. These findings are in disagreement with results from a previous study, where gluten-induced symptoms were found to be a good predictor of histological changes during a 4-week challenge.[9] A possible

limitation in generalizing from our findings may be the fact that we excluded subjects who had a history of severe gluten related symptoms. A prospective study including subjects on a gluten-free diet without prior diagnosis would have overcome this limitation, but the assessment of the morphological response might have become difficult due to a potentially higher number of subjects not being able to complete the challenge.

Taken together, this study demonstrates that a 14-day gluten challenge has inadequate sensitivity if villous blunting or increased coeliac disease specific antibody levels are used as outcome parameters. Repeat biopsies taken before and after a short gluten challenge can increase the sensitivity of the test, but not enough. Longer duration of the gluten challenge is required. Aiming for a workup that is based on a short-duration gluten challenge, the less invasive blood test based on HLA-DQ:gluten tetramers in a flow cytometric assay seems to be a sensitive biomarker that should be explored further.

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Contributors:

Study concept and design (KEAL, VKS, GIS, LMS), acquisition of data (VKS, HMR, LFR, SDK), analysis and interpretation of data (all authors), drafting of the manuscript (VKS, LMS), critical revision of the manuscript for important intellectual content (all authors), statistical analysis (VKS, LFR, GIS), obtained funding (KEAL, LMS), administrative (VKS, GIS, KEAL), technical and material support (VKS, GIS), study supervision (KEAL, LMS).

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FIGURE LEGENDS

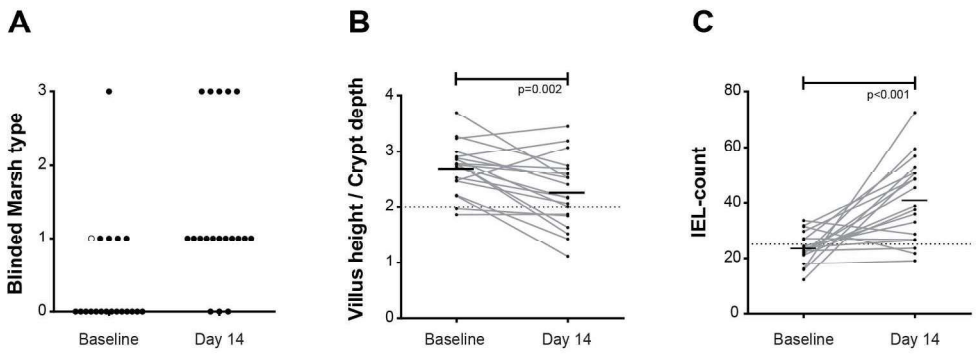
Figure 1: A small proportion of biopsies had villous blunting equivalent to Marsh type 3 or Vh/Cd < 2.0 at the end of challenge. (A) The blinded evaluation of the Marsh type for 20 participants at baseline and 19 participants on day 14 of gluten challenge; one participant (open circle) did not complete gluten challenge. (B) The villous height to crypt depth ratio (Vh/Cd) at baseline and day 14 of gluten challenge. (C) IEL-count in biopsies at baseline and on day 14 of gluten challenge. The dotted lines are drawn along commonly used cut-offs; Vh/Cd = 2 in panel B and IEL-count = 25 in panel C. Short horizontal lines indicate average. P-values were calculated by paired t-tests.

Figure 2: Weak antibody responses in serum until day 28 of gluten challenge, and several cytokines had significant elevations of plasma concentrations in the initial hours after gluten challenge. (A) Anti-TG2 IgA and (B) anti-DGP IgG levels for 19 participants at four time points during gluten challenge. The upper dotted line shows the positive cut-off and the stippled line below is drawn at the lower detection limit (LDL). The numerical value of LDL equals 1 in panel A, and 5 in panel B. Values lower than LDL were assigned half value of LDL. (C) Spider plot of median fold change in concentration for significantly elevated cytokines at the peak time point analysed from blood drawn at the first day of challenge from 19 participants.

Figure 3: HLA-DQ:gluten tetramer-binding gut-homing effector-memory $CD4^+$T cells (HLA-DQ:gluten-tetramer$^+\beta7^+T_{EM}$) in blood increase in frequency on day 6 of gluten challenge. (A) Blood HLA-DQ:gluten-tetramer$^+\beta7^+T_{EM}$ / 10^6 $CD4^+$ cells for 15 participants at baseline and day 6. If no cells were detected, value 0.1 was assigned. (B) CD38 expression in 10 subjects for baseline and day 6 in HLA-DQ:gluten-tetramer$^+\beta7^+T_{EM}$ on the left side of the panel and

HLA-DQ:gluten tetramer-negative $\beta7^{+}T_{EM}$ from the same subjects on the right side. Short horizontal lines indicate median. P-values were calculated by the Wilcoxon signed rank test.

Figure 4: Symptoms increased during the gluten challenge. (A) The Celiac Symptom Index (CSI) (range 16 – 80), scored at baseline and at end of gluten challenge (day 14) for all 20 participants. Short horizontal lines indicate medians. P-values were calculated by the Wilcoxon signed rank test. (B) Symptoms on day 1 of gluten challenge were scored by visual analogue scale (VAS) immediately prior to (baseline), and 2, 4 and 6 hours after gluten challenge. Results for three of 20 participants were excluded list wise due to missing values. The p-value was calculated by Friedman's test with post-hoc Dunn's adjustment.



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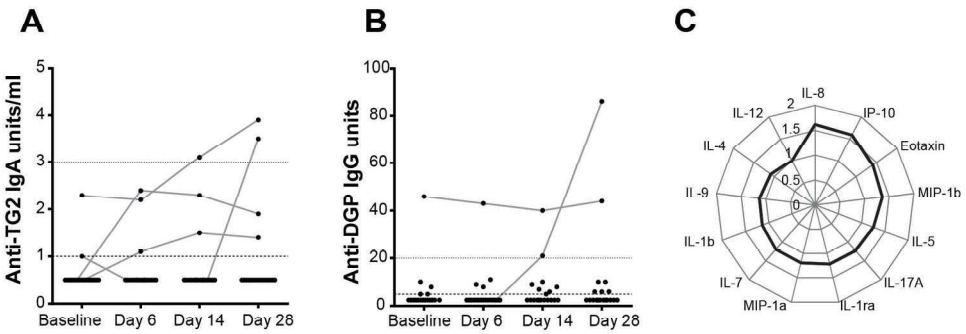

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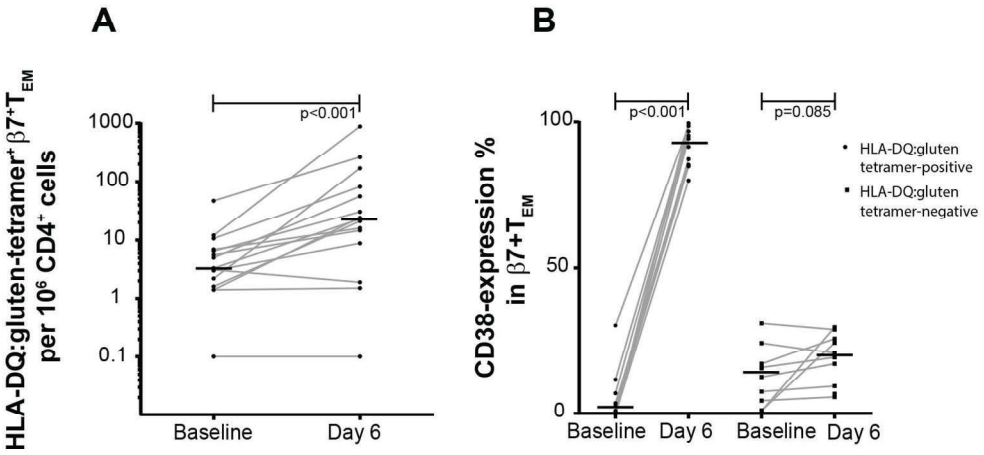

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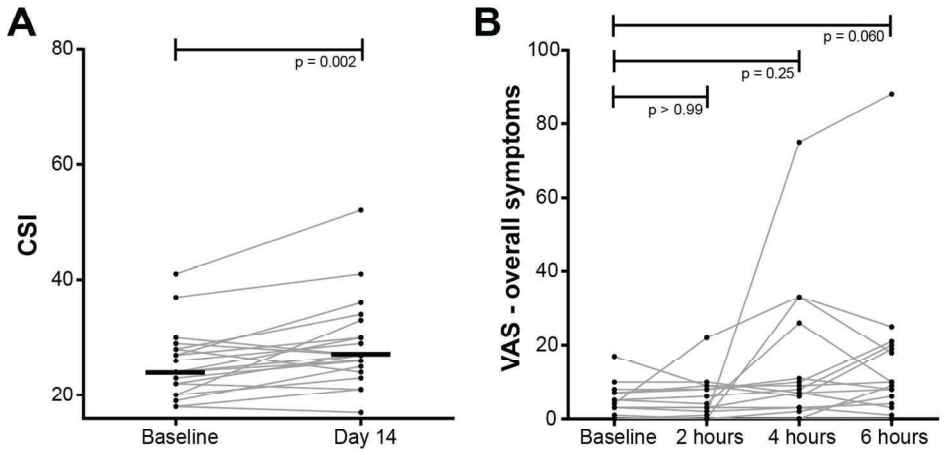

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SUPPLEMENTAL METHODS

Recruitment

We recruited participants both by direct invitation and announcement. The announcement was made on hospital employee web sites and in fora managed by a patient interest organization for coeliac disease. A total number of 78 subjects were invited or screened, and 20 were included. Among the 58 excluded subjects; 35 did not accept the invitation, 18 did not meet criteria and five had other reasons for not participating (significant comorbidity or travel distance).

Duodenal histopathology

The biopsies were fixated in formalin. Paraffin-embedded sections were stained with haematoxylin and eosin. Mucosal remission status at baseline was initially determined by non-blinded routine biopsy assessment of Marsh type. Subsequently, the slides were de-identified using a slide scanner (Pannoramic MIDI, 3DHistech, Budapest, Hungary) and the image files were exported to Coeliac Slide Viewer (JiLab Inc., Tampere, Finland). An experienced gastrointestinal pathologist (HMR) performed the morphometric measurements, cell counting and establishment of Marsh types, blinded for participant identity and study visit. At least three adequately oriented crypts were required, indicating a section plane perpendicular to the mucosal surface, for valid morphometric measurements in each set of biopsies. Mean Vh/Cd was computed by dividing villous height by the depth of an adjacent crypt in all measurable and adequately oriented villous-crypt pairs in each set of biopsies, using the built-in measurement tool in Coeliac Slide Viewer. Quantification of IEL was performed by counting all, and at least 100 contiguous enterocytes, in at least 4 villi, and the results were reported as a global mean number of IEL per 100 enterocytes (IEL-count).

Frequency estimation of gluten-specific T cells using HLA-DQ:gluten tetramers

HLA-DQ:gluten tetramers consisted of recombinant biotinylated HLA-DQ molecules with peptide epitopes linked to the N-terminus of the DQβ-chain in complex with phycoerythrin (PE)-conjugated streptavidin. DQ2.5 (DQA1*05:01/ DQB1*02:01) molecules representing the epitopes HLA-DQ2.5-glia-α1a (QLQPFPQPELPY, with underlined 9-mer core sequence), DQ2.5-glia-α2 (PQPELPYPQPE), DQ2.5-glia-ω1 (QQPFPQPEQPFP) and DQ2.5-glia-ω2 (FPQPEQPFPWQP) were produced in baculovirus expression system. HLA-DQ8 (DQA1*03:01/DQB1*03:02) molecules were produced in stably transfected S2 cells representing the DQ8-glia-α1 (SGEGSFQPSQENPQ) and DQ8-glia-γ1b (FPEQPEQPYPEQ) epitopes. Peripheral blood mononuclear cells were prepared by gradient centrifugation and incubated with an equal mixture of the four HLA-DQ2.5 tetramers or two HLA-DQ8 tetramers (10 µg/ml of each tetramer) for 30-45 min at room temperature. Anti-PE microbeads and magnetic columns (autoMACS® Pro Separator, Milenyi Biotec, Bergisch Gladback, Germany) were used to enrich for HLA-DQ:gluten tetramer-binding cells prior to staining with a mixture of monoclonal antibodies; CD45RA-PE-Cy7 and CD3-eVolve 605 (both from eBioscience, Thermo Fisher Scientific, Waltham, MA), CD11c-Pacific Blue (PB) and CD4-APC-H7 (both from BD Biosciences, San Jose, CA) and CD62L-PerCP/Cy5.5, integrin β7-APC, CD14-PB, CD19-PB, CD56-PB (all from BioLegend, San Diego, CA). Some of the samples were also stained with CD38-FITC (eBioscience, Thermo Fisher Scientific, Waltham, MA).

Patient reported outcomes

The CSI comprises 16 questions. The VAS is a linear scale, and scores were done for pain, bloating, flatulence, nausea, stool consistency and overall symptoms. Weekly averages were calculated from daily VAS-scores. VAS-scores for overall symptoms were done on first day of gluten challenge; at baseline, 2, 4 and 6 hours following the first intake of gluten.

Statistics

For approximately normally distributed data, we performed a paired t-test or ANOVA with a post hoc Dunnett's test for multiple comparisons relative to baseline as significance tests. For

non-Gaussian data, we used the Wilcoxon signed rank test for paired data, or Friedman's test with a post hoc Dunn's adjusted test for multiple comparisons relative to baseline. Relationships between outcome and baseline variables were examined by non-parametric correlations (Spearman rho). The significance level was $p<0.05$ adjusted for multiple testing. Missing values were excluded list wise. Data points with values below the lower detection limit (LDL) were assigned half the value of the LDL.

Supplementary tables and figure legends:

Inclusion criteria	Exclusion criteria
Coeliac disease verified by either positive biopsy or positive serology before start of gluten-free diet if biopsy is yet not done and is expected to be positive after a challenge	Pregnant or breast feeding
	Woman in fertile age not taking adequate contraceptive measures
	Use of immune suppressive medication for the last three months
	Chronic (other gastrointestinal or systemic disease) or severe acute infection
Following a gluten-free diet for at least 6 months	Strong reaction to small amounts of ingested gluten
Age between 18 and 80 years	Allergy to sesame seeds, pecan or macadamia nuts
Given written informed consent for participation	Positive anti-transglutaminase 2 IgA or duodenal biopsy (Marsh type 2 or 3) at baseline

Supplementary table 1: Inclusion and exclusion criteria.

Ingredients	50 g muesli bar
Maple syrup	7.7 g
Rice malt	7.4 g
Soft brown sugar	7.1 g
Sesame seeds	3.4 g
Pecans	3.4 g
Quinoa flakes	2.3 g
Pepitas	2.3 g
Puffed quinoa	1.6 g
Macadamia oil	1.6 g
Rice puffs	1.3 g
Gluten flour	7.6 g
White chia seeds	4.8 g

Supplementary table 2: List of ingredients in a 50 g muesli bar

ID	Age, years	Gen-der	HLA geno-type	BMI, m /kg^2	GFD, mont hs	CSI base-line	CSI Day 14	VAS overall symp-toms base-line	VAS overall symp-toms 2 h	VAS overall symp-toms 4 h	VAS overall symp-toms 6 h
CD442	57	M	DQ2.5 /DQ2.5	25.1	176	20	33	0	1	75	88
CD1178	55	F	DQ2.5 /DQ8	21.8	26	24	30	5	6	8	20
CD1284	34	F	DQ2.5 /X	26.9	183	22	21	0	0	0	0
CD1294	23	F	DQ8 /DQ7	20.6	216	22	27	10	10	7	8
CD1295	42	F	DQ2.5 /X	29.0	165	26	29	2	15	ND	12
CD1296	55	F	DQ2.5 /X	23.1	97	28	24	7	8	11	8
CD1298	42	F	DQ2.5 /X	22.1	42	24	26	0	0	0	ND
CD1299	21	F	DQ2.5 /X	25.3	113	29	27	3	2	3	1
CD1300	60	F	DQ2.5 /X	23.5	338	20	23	8	8	10	21
CD1302	26	F	DQ8/8	18.0	170	27	36	5	4	26	10
CD1303	78	F	DQ2.5 /X	25.4	131	18	21	0	0	0	0
CD1339	60	F	DQ2.5/ DQ2.2	20.2	180	37	41	0	0	2	6
CD1340	60	M	DQ2.5 /DQ2.5	29.4	34	41	52	17	9	9	10
CD1342	26	F	DQ2.5 /X	23.2	60	27	30	11	6	2	ND
CD1343	25	M	DQ8 /DQ2.2	22.3	239	19	25	7	9	6	19
CD1351	23	F	DQ2.5 /X	23.2	32	30	27	3	3	7	3
CD1353	36	F	DQ2.5 /X	21.1	105	24	27	1	0	33	18
CD1366	42	M	DQ2.5 /X	22.0	473	23	26	3	3	3	4
CD1378	41	F	DQ2.5 /X	20.1	95	18	17	4	22	33	25
CD1379	26	F	DQ2.5 /DQ8	34.8	147	28	34	0	0	0	9

ID	Marsh type base-line	Marsh type day 14	Vh/Cd base-line	Vh/ Cd Day 14	IEL base-line	IEL Day 14	Tetra-mer /10^6 CD4$^+$ base-line	Tetra-mer /10^6 CD4$^+$ Day 6	CD38% in tetra-mer base-line	CD38% in tetra-mer Day 6
CD442	3	3	1.86	1.87	26.8	56.9	46.8	260.6	30.2	98.6
CD1178	0	1	2.88	2.52	24.4	33.1	3.1	1.9	ND	ND
CD1284	1	1	2.78	2.70	27.2	26.8	5.2	ND	ND	ND
CD1294	0	0	2.47	3.07	22.7	23.6	0.1	ND	ND	ND
CD1295	0	3	2.20	1.11	22.4	45.4	49.5	ND	ND	ND
CD1296	0	3	2.77	1.63	21.9	48.6	1.4	24.2	ND	ND
CD1298	0	0	3.27	2.75	17.9	19.1	1.4	1.5	ND	ND
CD1299	0	1	3.23	3.44	23.9	26.5	5.0	55.9	ND	ND
CD1300	0	3	2.21	1.43	15.8	52.9	12.1	881.4	ND	ND
CD1302	0	1	2.46	2.06	21.2	37.5	3.0	8.8	1.0	84.7
CD1303	1	1	2.76	2.59	33.6	28.8	5.6	14.6	ND	91.5
CD1339	1	1	1.97	1.84	29.7	48.4	3.3	22.9	11.7	96.8
CD1340	0	1	2.52	2.17	21.4	59.5	2.2	169.4	0.0	99.5
CD1342	0	1	2.86	2.01	16.2	72.4	1.6	21	2.6	87.2
CD1343	0	3	2.78	1.52	12.4	50.6	6.9	16	0.0	79.9
CD1351	0	1	3.00	2.40	31.8	50.8	2.4	ND	3.6	85.2
CD1353	0	1	2.73	2.16	22.3	38.8	10.7	81	0.7	94.3

CD1366	1	0	2.91	3.19	31.5	21.7	0.0	0	ND	ND
CD1378	1	ND	2.57	ND	34.1	ND	23.5	ND	30.3	ND
CD1379	0	1	3.69	2.53	23.4	36.0	6.5	30.8	7.0	95.3

ID	TG2 base-line, U/mL	TG2 Day 6, U/mL	TG2 Day 14, U/mL	TG2 Day 28, U/mL	DGP base-line, U	DGP Day 6, U	DGP Day 14, U	DGP Day 28, U
CD442	2.3	2.2	3.1	3.9	8	9	10	10
CD1178	<1	<1	<1	<1	10	11	9	10
CD1284	<1	<1	<1	<1	<5	<5	<5	<5
CD1294	<1	<1	<1	<1	<5	<5	<5	<5
CD1295	<1	<1	<1	<1	<5	<5	<5	<5
CD1296	<1	<1	<1	<1	<5	<5	<5	<5
CD1298	<1	<1	<1	<1	<5	<5	<5	<5
CD1299	<1	<1	<1	<1	<5	<5	<5	<5
CD1300	<1	<1	<1	<1	<5	<5	<5	<5
CD1302	<1	<1	<1	<1	<5	<5	<5	<5
CD1303	<1	<1	<1	<1	46	43	40	44
CD1339	<1	<1	<1	<1	5	8	7	6
CD1340	<1	<1	<1	3.5	<5	<5	21	86
CD1342	<1	2.4	2.3	1.9	<5	<5	5	<5
CD1343	<1	<1	<1	<1	<5	<5	<5	6
CD1351	<1	1.1	1.5	1.4	5	<5	6	6
CD1353	<1	<1	<1	<1	<5	<5	8	<5
CD1366	<1	<1	<1	<1	<5	<5	<5	<5
CD1378	<1	ND	ND	ND	<5	ND	ND	ND
CD1379	1	<1	<1	<1	<5	<5	<5	<5

ID	IL-8 base-line, pg /mL	IL-8 2 h, pg /mL	IL-8 4 h, pg/mL	IL-8 6 h, pg/mL	MIP-1β base-line pg/mL	MIP-1β 2 h, pg/mL	MIP-1β 4 h, pg/mL	MIP-1β 6 h, pg/mL	IP10 base-line, pg/mL	IP10 2 h, pg/mL	IP10 4 h, pg/mL	IP10 6 h, pg/mL	Eo-taxin Base-line pg/mL	Eo-taxin 2 h pg/mL	Eo-taxin 4 h, pg/mL	Eo-taxin 6 h, pg/mL
CD442	12.69	14.56	78.76	84.39	58.76	58.56	388.6	244.16	359.81	423.33	1025.9	2134.0	99.81	106.7	135.9	141.2
CD1178	15.32	10.85	12.48	22.62	47.05	52.13	51.51	49.66	456.09	591.36	563.36	478.78	33.64	36.92	33.64	44.03
CD1284	10.85	10.85	11.66	18.16	50.39	54.05	54.05	67.19	223.8	249.36	246.07	324.77	34.88	35.7	36.11	55.94
CD1294	10.04	9.22	12.48	12.48	42.55	46.84	41.45	44.39	488.97	526.22	395.53	434.63	28.1	31.96	40.12	40.12
CD1295	16.13	22.21	98.71	36.35	20.31	45.99	69.51	29.07	552.98	710.55	922.21	949.78	36.11	36.11	42.48	31.96
CD1296	22.62	41.59	27.87	24.24	39.56	48.1	36.26	34.63	327.45	403.12	376.36	377.65	49.34	76.61	58.09	55.94
CD1298	21.4	20.59	14.1	16.54	21.44	20.88	21.44	11.95	84.38	93.51	87.46	92.51	32.8	32.8	31.12	31.12
CD1299	14.1	16.54	27.07	10.85	26.66	28.82	26.53	33.21	104.28	141.27	153.75	225.68	32.8	34.47	51.56	34.05
CD1300	10.85	9.22	22.21	20.59	21.16	23.63	39.67	30.55	473.06	492.84	940.87	970.36	41.7	46.32	51.19	58.8
CD1302	15.73	16.54	33.93	27.07	34.63	30.18	72.07	49.14	474.27	399.33	487.77	803.56	26.78	24.99	28.54	30.26
CD1303	13.7	11.66	18.88	23.74	82.49	68.68	91.2	97.23	709.46	647.47	627.59	734.2	90.82	71.26	95.16	110.1
CD1339	10.79	7.92	15.8	12.06	43.14	41.31	71.08	57.16	200.1	189.03	244.13	242.21	41.99	39.8	95.74	50.83
CD1340	13.63	12.06	17.04	22.23	56.21	60.55	77.44	79.17	542.76	528.02	524.19	666.34	54.92	57.61	64.5	72.16
CD1342	17.04	17.04	21.32	21.62	48.36	44.44	49.97	53.43	742.96	717.4	705.74	795.41	39.8	41.99	47.7	52.2
CD1343	10.48	18.88	27.05	56.58	53.97	67.19	109.36	182.28	338.15	414.74	477.48	1063.48	40.53	54.25	57.61	66.44
CD1351	6.29	7.59	16.11	10.79	30.24	32.16	81.67	39.38	110.79	99.28	140.32	193.41	23.29	27.45	32.23	39.06

CD1353	11.43	10.16	14.56	16.73	34.2	32.56	43.79	37.65	420.75	350.65	404.11	573.09	33.78	35.69	35.69	42.72
CD1366	8.88	9.52	10.48	6.29	30.08	24.77	23.21	19.46	222.84	171.3	127.22	125.75	33.78	33.78	31.45	32.23
CD1378	6.94	8.88	27.35	17.34	24.6	26.96	94.02	49.28	236.76	270.68	801.76	805.83	26.63	32.23	49.1	49.1
CD1379	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Supplementary table 3: Participant characteristics and data.

Note: F, female; M, male; h, hours; U, units; BMI, body mass index; tetramer, HLA-DQ: gluten tetramer+β7+ T_{EM} in blood

Time point	Parameter	IL1b	IL-1ra	IL-4	IL-5	IL-7	IL-8	IL-9	IL-12	IL-17A	Eotaxin	IP-10	MIP-1α	MIP-1β
2 hours	Concentration change, P-value[a]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Symptom correlation, P-value[b]	n.s.	n.s.	n.s.	n.s.	n.s.	0.036	n.s.	n.s.	n.s.	0.021	n.s.	n.s.	0.003
	Symptom correlation, rho[c]	0.102	0.245	0.118	0.115	-0.061	0.561	0.352	0.097	0.138	0.596	0.500	-0.098	0.677
4 hours	Concentration change, P-value[a]	0.009	0.036	0.014	0.043	0.030	<0.001	0.036	0.004	0.030	0.003	n.s.	0.008	0.006
	Symptom correlation, P-value[b]	n.s.	n.s.	n.s.	n.s.	n.s.	0.015	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.015
	Symptom correlation, rho[c]	-0.207	-0.004	-0.225	-0.226	-0.041	0.617	-0.044	0.013	-0.002	0.315	0.522	-0.200	0.618
6 hours	Concentration change, P-value[a]	n.s.	n.s.	n.s.	0.014	n.s.	0.003	n.s.	n.s.	n.s.	<0.001	<0.002	n.s.	0.030
	Symptom correlation, P-value[b]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Symptom correlation, rho[c]	-.219	.186	-.156	.010	-.038	.430	-.026	.081	-.196	.114	.453	-.130	.457

Supplementary table 4: Significantly elevated cytokines and Spearman correlation to symptoms at two, four and six hours after gluten challenge

Note. ns, not significant. [a]Significance tests were done with Friedman's nonparametric test for repeated measures and Dunn's adjusted multiple comparisons test compared to baseline. [b]Change in symptoms (relative to baseline) and change in cytokine concentrations (relative to baseline) were assessed by Spearman correlations for each time point. P-value is adjusted for three repeated tests per cytokine (Bonferroni). [c]Spearman correlation coefficient

for change in symptoms relative to baseline and relative change in cytokine concentrations for each time point.

Spearman correlations		Vh/Cd fold change (day 14)	IEL fold change (day 14)	Anti-TG2 fold change (day 28)	Anti-DGP fold change (day 28)	CSI fold change (day 14)	Tetra-mer baseline	Vh/Cd baseline	IEL baseline	Day 1 VAS fold change (peak)	Fold change IL8 (4 hours)	Fold change MIP-1β (4 hours)
HLA-DQ:gluten tetramer test fold change (Day 6)	Correlation Coefficient	0.311	0.621	0.413	0.219	-0.243	0.164	-0.407	-0.329	-0.022	0.204	0.116
	P-value	>0.99	0.134	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
	N	15	15	15	15	15	15	15	15	15	14	14
Vh/Cd fold change (day 14)	Correlation Coefficient		0.691	-0.086	0.076	-0.088	0.352	-0.011	-0.602	0.359	0.319	0.397
	P-value		**0.010**	>0.99	>0.99	>0.99	>0.99	>0.99	0.064	>0.99	>0.99	>0.99
	N		19	19	19	19	19	19	19	19	18	18
IEL fold change (day 14)	Correlation Coefficient			0.419	0.490	0.200	0.291	-0.368	-0.695	0.389	0.461	0.451
	P-value			0.744	0.330	>0.99	>0.99	>0.99	**0.010**	0.995	0.540	0.603
	N			19	19	19	19	19	19	19	18	18
Anti-TG2 fold change (day 28)	Correlation Coefficient				0.521	0.026	-0.192	-0.197	-0.041	-0.171	0.198	0.288
	P-value				0.222	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
	N				19	19	19	19	19	19	18	18
Anti-DGP fold change (day 28)	Correlation Coefficient					0.240	0.073	-0.217	-0.119	0.310	0.378	0.485
	P-value					>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	0.415
	N					19	19	19	19	19	18	18
CSI fold change (day 14)	Correlation Coefficient						0.160	-0.338	-0.278	0.268	0.165	0.279
	P-value						>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
	N						20	20	20	20	19	19
Tetramer baseline	Correlation Coefficient							-0.397	0.091	0.607	0.706	0.740
	P-value							0.829	>0.99	**0.045**	**0.007**	**0.003**
	N							20	20	20	19	19
Vh/Cd baseline	Correlation Coefficient								0.033	-0.376	-0.502	-0.549
	P-value								>0.99	>0.99	0.286	0.149
	N								20	20	19	19
IEL baseline	Correlation Coefficient									0.072	0.075	0.093
	P-value									>0.99	>0.99	>0.99
	N									20	19	19

Supplementary table 5: Non-parametric correlation matrix for some response parameters and baseline levels.

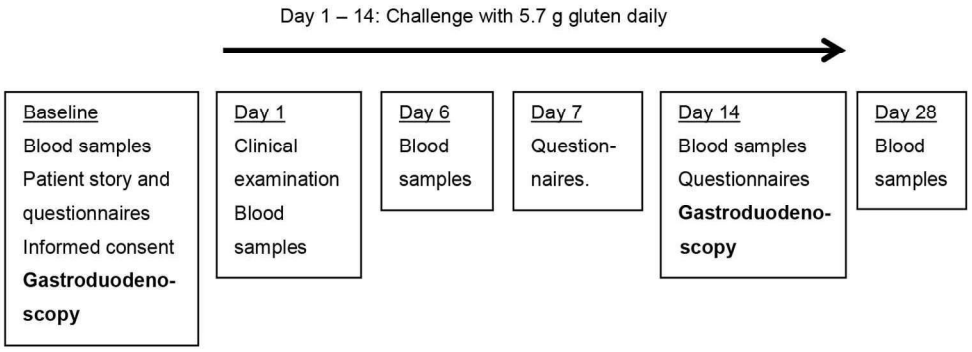
Note. Fold change for response parameters is calculated by dividing the endpoint value (at the time point in parenthesis) by the baseline value. The P-value for correlations is adjusted for 10 repeated tests (Bonferroni) and significant P-values are highlighted in bold font.

Supplemental figure legends:

Supplemental figure 1: The timeline of the study.

Supplemental figure 2: Gating strategy for $CD4^+$ effector-memory gut-homing HLA-DQ:gluten tetramer-binding T cells. Peripheral blood mononuclear cells were gated for lymphocytes, single cells, $CD3^+$, excluded for CD11c, CD14, CD19 and CD56 (dump channel), $CD4^+$, HLA-DQ:gluten-$tetramer^+$, $CD45RA^-$, $CD62L^-$, integrin $\beta7^+$.

Supplemental figure 3: Symptoms related to stool consistency during week 1 and flatulence on week 2 of gluten challenge. Different symptoms, as specified on the y-axes, were scored daily. (A – F) Weekly averages based on daily visual analogue scale (VAS) scores for different symptoms before gluten challenge (baseline), and during the first and the second week of gluten challenge. VAS is a linear scale ranging from 0 to 100.



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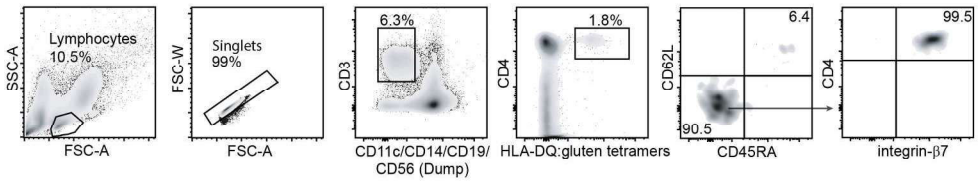

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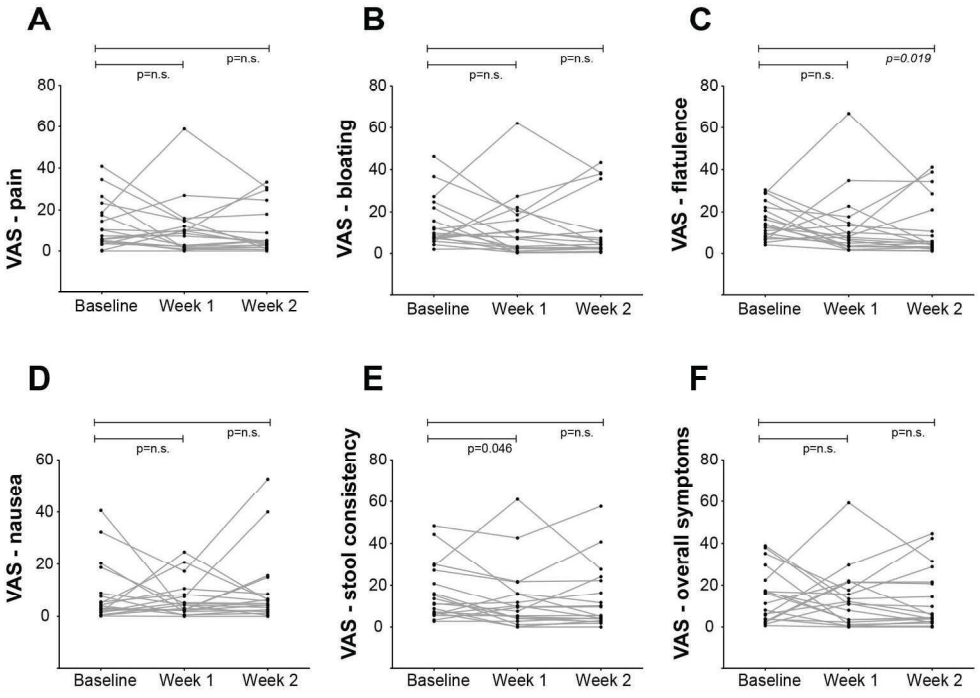

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