ORIGINAL ARTICLE

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 5 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 9 of 19 subjects. Interleukin-8 plasma concentration increased by more than 100% after 4 hours in 7 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 patients with coeliac disease (sensitivity ≈25%–50%). Increase in $CD4^+$ effector-memory gut-homing HLA-DQ:gluten tetramer-binding T cells in blood 6 days after gluten challenge is a more sensitive and less invasive biomarker that should be validated in a larger study.

Trial registration number

Significance of this study

What is already known on this subject?
- Many subjects maintain a gluten-free diet without prior work-up of coeliac disease.
- For subjects in this situation, a recommended work-up of coeliac disease requires a gluten challenge for 2 to 8 weeks, followed by a duodenal biopsy. This procedure may cause unacceptable symptoms in some patients.
- The recommendation of a 2-week gluten challenge is based on limited evidence, and the sensitivity of this procedure is not well validated.

What are the new findings?
- A 2-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 twofold change in plasma concentration of interleukin (IL)-8 and macrophage inflammatory protein (MIP)-1β in some of the subjects with coeliac disease in remission.
- A twofold change in gluten-specific T-cell response in blood, measured by HLA-DQ:gluten tetramers, was detected after 6 days of gluten challenge in a majority of subjects with coeliac disease in remission.

How might it impact on clinical practice in the foreseeable future?
- This study lowers expectations of a positive duodenal histology after a 2-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 characterised 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 recognise DGPs 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 lifelong 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 2 weeks, prolonged to 8 weeks if possible, followed by duodenal biopsy.[1 7 8] The recommended duration of minimum 2 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, and 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 ratio (Vh/Cd) and 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 ELISPOT after incubation of mononuclear blood cells with gluten[16 17] or direct detection of gluten-specific T cells in mononuclear blood cells with the use of HLA-DQ:gluten tetramers and flow cytometry.[18 19] Other potential parameters for prediction of disease-specific inflammation may include cytokine production in the early phases of a gluten challenge.[20]

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 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 online supplementary table S1. For further details on the participant recruitment, see online supplementary methods.

Gluten challenge protocol

A baseline duodenal biopsy was taken, in most cases 1 to 2 weeks before the onset of challenge, to confirm remission in all participants (online supplementary 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 oligosaccharides, disaccharides monosaccharides and polyols (online supplementary table S2). 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 online supplementary methods for details. Vh/Cd >2 was considered normal.[10 21] An IEL count of 25 was used as cut-off between Marsh type 0 and 1.[21]

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, San Diego, California, USA) 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, California, USA).

At the first day of challenge, plasma samples for cytokine determination were collected prior to gluten challenge, and then 2, 4 and 6 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, California, USA). Data analysis was done with Bio-Plex MAGPIX Multiplex Reader and Bio-Plex Manager 6.1 software (Bio-Rad).

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 19 22] 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 multimerisation 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 online supplementary methods. The cells were analysed by flow cytometry and gated for $CD4^+CD3^+CD11c^-CD14^-CD19^-CD56^-CD45RA^-CD62L^-$integrinβ$7^+$HLA-DQ:gluten tetramer$^+$ (HLA-DQ:gluten tetramer$^+$β7+T_{EM}) (see online supplementary figure S2). The number of HLA-DQ:gluten tetramer$^+$β7+T_{EM} was normalised 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 online supplementary methods for further details.

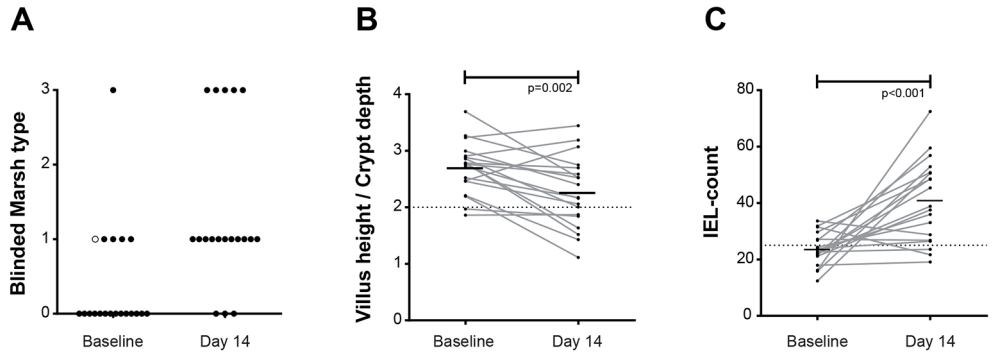

Figure 1 A small proportion of biopsies had villous blunting equivalent to Marsh type 3 or Vh/Cd <2.0 at the end of challenge. (A) The blinded evaluation of the Marsh type for 20 participants at baseline and 19 participants on day 14 of gluten challenge; one participant (open circle) did not complete gluten challenge. (B) The villous height to crypt depth ratio (Vh/Cd) at baseline and day 14 of gluten challenge. (C) Intraepithelial lymphocyte (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-test.

Statistics

Statistical analysis was done on GraphPad Prism V. 7.02 (GraphPad Software, La Jolla, California, USA) and SPSS (IBM SPSS Statistics V. 22.0, North Castle, New York, USA). Power transformations were applied where necessary. See online supplementary 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 body mass index 23.8 kg/m^2 (SD 3.9) (online supplementary table S3). 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 gastroduodenoscopies. 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 3 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 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).

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 response 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 H&E-stained biopsies by others,[15] we were able to detect response in 12 of 19 participants (table 1). 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 after start of gluten challenge (table 1). Similarly, two participants were positive for anti-DGP IgG on 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 4 or 6 hours after gluten challenge compared with baseline (figure 2C). Three cytokines had a highly significant increase (p<0.001); interleukin (IL)-8, interferon-γ inducible protein (IP)-10 and eotaxin with peak median fold changes of 1.6, 1.6 and 1.4 respectively, all peaking at 6 hours (online supplementary table S3). 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), granulocyte macrophage colony stimulating factor (GM-CSF) (LDL=1.2 pg/mL), monocyte chemoattractant protein (MCP)-1 (LDL=5.04 pg/mL) and vascular endothelial growth factor (VEGF) (LDL=9.36 pg/mL). Other measured cytokines did not show any significant change from baseline levels: IL-10, IL-13, fibroblast growth factor (FGF) basic, platlet derived growth factor (PDGF)-bb, interferon-γ, G-CSF, tumor necrosis factor (TNF)-α and RANTES.

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

An arbitrary cut-off of twofold change was defined for HLA-DQ:gluten tetramer$^+$β7$^+$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 on day 6 were not available for four participants due to technical reasons, and excluded for one participant due to immune suppressive

Table 1 Response parameters in 14-day gluten challenge

ID	HLA-DQ:gluten tetramer test fold change day 6	IEL fold change day 14	Vh/Cd difference day 14	Interleukin-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

Response parameters in the top row are sorted by decreasing sensitivity of response, showing HLA-DQ:gluten tetramer test 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.
DGP, deamidated gliadin peptide; HLA-DQ:gluten tetramer test, HLA-DQ:gluten tetramer$^{+}\beta7^{+}T_{EM}/10^{6}CD4^{+}$ cells; IEL, intraepithelial lymphocyte; ND, not done; TG2, transglutaminase 2; U, units.

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 to 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 significant difference ($p=0.085$) from baseline to day 6.

Symptoms increased during gluten challenge

Symptoms, as scored by the CSI, increased significantly ($p=0.002$) from baseline to the end of challenge from a median

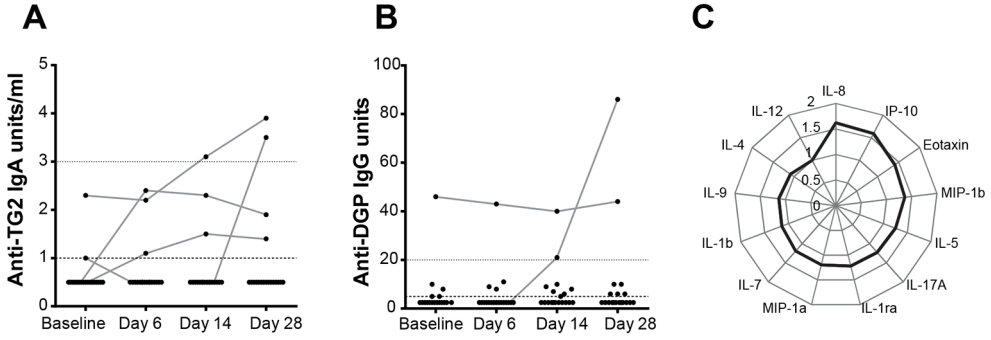

Figure 2 Weak antibody responses in serum until day 28 after start of 14-day gluten challenge, and several cytokines with significant elevations of plasma concentrations in the initial hours after gluten challenge. (A) Anti-transglutaminase 2 (TG2) IgA and (B) anti-deamidated gliadin peptide (DGP) IgG levels for 19 participants at four time points. 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.

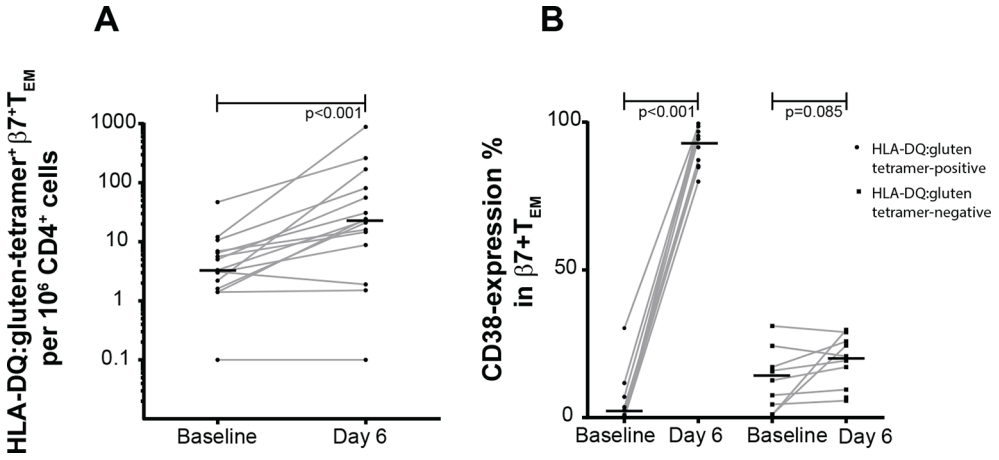

Figure 3 HLA-DQ:gluten tetramer-binding gut-homing effector-memory CD4+ T cells (HLA-DQ:gluten tetramer$^+\beta7^+T_{EM}$) in blood increase in frequency on day 6 of gluten challenge. (A) Blood HLA-DQ:gluten tetramer$^+\beta7^+T_{EM}$/10^6 CD4+ cells for 15 participants at baseline and on day 6. If no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

score of 24 (IQR 7) to 27 (IQR 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) (online supplementary figure S3). 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 postchallenge, compared with 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 on day 1 of gluten challenge (online supplementary table S4). 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-associated response of these cytokines, we could find a twofold change in concentration, chosen as an arbitrary cut-off, in 7 of 19 participants for IL-8 and in 6 of 19 for MIP-1β (table 1).

Correlation between outcome parameters and baseline parameters

Fold change in level of blood HLA-DQ:gluten tetramer$^+\beta7^+T_{EM}$ (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 (online supplementary table S5). 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$^+\beta7^+T_{EM}$ 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

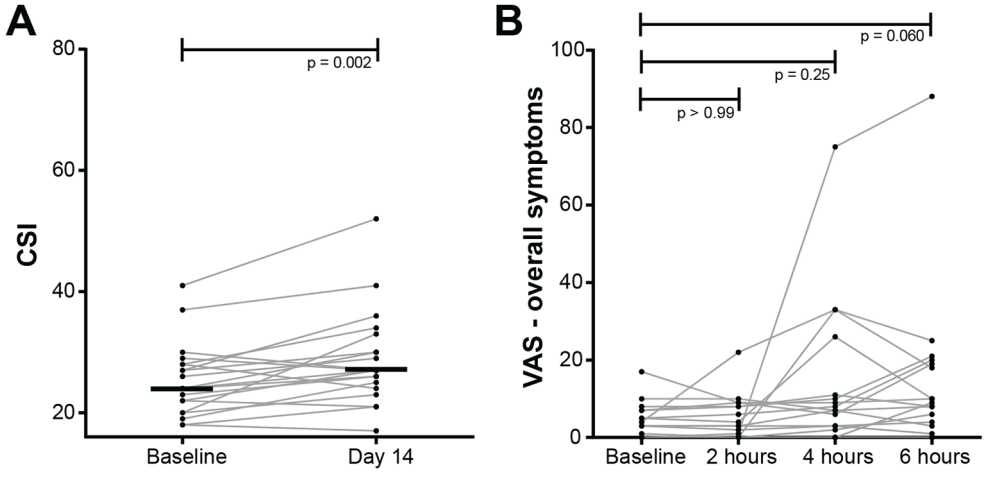

Figure 4 Symptoms increased during the gluten challenge. (A) The Celiac Symptom Index (CSI) (range 16–80) scored at baseline and at end of gluten challenge (day 14) for all 20 participants. Short horizontal lines indicate medians. p Values were calculated by Wilcoxon signed-rank test. (B) Symptoms on 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 3 of 20 participants were excluded listwise due to missing values. The p value was calculated by Friedman's test with post hoc Dunn's adjustment.

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 with 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 of gluten-free diet prior to gluten challenge (average 65 vs 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 vs bread) or sources of gluten were qualitatively different. The dose of gluten in Leffler *et al* was 3 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 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, that is, an absolute change in Vh/Cd of 0.4 and an IEL change of about 50% in H&E-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 has 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 seropositivity 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 seropositivity after 14-day gluten challenge, in contrast to 55% in Leffler *et al*. Differences in baseline remission status between the current and the Leffler *et al* study, in addition to differences in cut-offs and dynamic range between the different assays used, could potentially explain the different degrees of seropositivity in their compared with 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$_{EM}$/10^6 CD4$^+$ cells. IL-8 and MIP-1β are known to be proinflammatory 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.[20 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-1α) in blood, 4 hours after intradermal 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$^+$β7$^+$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$^+$β7$^+$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 ELISPOT 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,[22] and was therefore preferred to the ELISPOT test in the current study. Moreover, we found that the expression of the activation marker CD38 by HLA-DQ:gluten tetramer$^+$β7$^+$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 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] and 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 3-day challenge, and not continuous challenge in 6 days as was done in the current protocol, should suffice.[17–19] If undertaking a gluten challenge as part of the work-up, this 3-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 (fewer HLA-DQ:gluten tetramers and without the gut-homing marker integrin β7), 11 of 13 HLA-DQ2.5^{+}-treated patients with coeliac disease had HLA-DQ:gluten tetramer$^{+}T_{EM}/10^{6}CD4^{+}$cells≥1, while all 10 control subjects were below this cut-off.[22] 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 commercialisation 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$^{+}\beta7^{+}T_{EM}/10^{6}CD4^{+}$ cells on gluten challenge is striking. The reason for this large variation in response is currently unknown. However, the observation of lower than median baseline levels of HLA-DQ:gluten tetramer$^{+}\beta7^{+}T_{EM}/10^{6}CD4^{+}$ cells in the three non-responders that showed less than twofold change on day 6, may suggest 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 GI 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 generalising 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 diagnostic work-up 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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Competing interests Knut EA Lundin and Ludvig M Sollid are holders of a patent application on the detection of gluten-specific T cell by HLA-DQ:tetramers (EP20140789602). KEAL is an advisor to ImmusanT. LMS is an advisor to ImmusanT and is consultant to Celgene and Intrexon. Regeneron and ImmusanT have provided research grants to the research group of LMS.

Patient consent All data are de-identified. All patients have signed a separate consent form approved by the regional ethical committee of South-East Norway and have consented to publication in medical journal.

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