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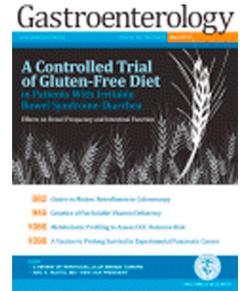
Vikas K. Sarna, Knut E.A. Lundin, Lars Mørkrid, Shuo-Wang Qiao, Ludvig M. Sollid, Asbjørn Christophersen

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## HLA-DQ–Gluten Tetramer Blood Test Accurately Identifies Patients With and Without Celiac Disease in Absence of Gluten Consumption

**Short title:** Blood based flow-cytometric test for celiac disease

**Authors:**

Vikas K. Sarna<sup>1,2</sup>, Knut E. A. Lundin<sup>2,3</sup>, Lars Mørkrid<sup>4</sup>, Shuo-Wang Qiao<sup>1,2,5</sup>, Ludvig M. Sollid<sup>1,2,5</sup>, Asbjørn Christophersen<sup>1,2</sup>

<sup>1</sup>Department of Immunology, Oslo University Hospital – Rikshospitalet, Norway.

<sup>2</sup>KG Jebsen Coeliac Disease Research Centre, University of Oslo, Norway.

<sup>3</sup>Department of Gastroenterology, Oslo University Hospital – Rikshospitalet, Norway.

<sup>4</sup>Department of Medical Biochemistry, Oslo University Hospital – Rikshospitalet, Norway.

<sup>5</sup>Centre for Immune Regulation, Oslo University Hospital – Rikshospitalet and University of Oslo, Norway.

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**Abbreviations:** AUROC, area under the receiver operating characteristic curve;  $\beta 7$ , integrin  $\beta 7$ ; [B-C; $\lambda$ ], Box-Cox power transformed; DGP, deamidated gliadin peptide; gluten-specific T cells, HLA-DQ–gluten-tetramer<sup>+</sup> integrin  $\beta 7$ <sup>+</sup> effector memory T cells; GFD, gluten-free diet; GS, self-reported gluten sensitive without celiac disease; HLA-DQ–gluten tetramer<sup>+/-</sup>, CD4<sup>+</sup>CD3<sup>+</sup>CD11c<sup>-</sup>CD14<sup>-</sup>CD19<sup>-</sup>CD56<sup>-</sup> and HLA-DQ–gluten-tetramer<sup>+/-</sup>; PBMC, peripheral blood mononuclear cells; ROC, receiver operating characteristic; TCD, treated celiac disease;

T<sub>EM</sub>, effector-memory T cells; TG2, transglutaminase 2; T<sub>N</sub>, naïve T cells; UCD, untreated celiac disease.

**Correspondence:**

Vikas K. Sarna, Department of Gastroenterology, Oslo University Hospital – Ullevål, Kirkeveien 166, 0450 Oslo, Norway.

**E-mail:** v.sarna@hotmail.com

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**Author contributions:**

Study concept (AC, LMS), study design (AC, VKS, KEAL, LM, LMS), acquisition of data (VKS), data analysis and interpretation (VKS, AC, LMS, SW), drafting the manuscript (VKS, AC, LMS, SWQ), critical manuscript revision (KEAL, LM), statistical analysis (LM, VKS), drafting study grant application and protocol optimization (AC), obtaining funding (LMS, KEAL), administrative (VKS, AC, SWQ), study supervision (AC, LMS, KEAL).

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## Abstract

**Background & Aims:** Celiac disease is characterized by HLA-DQ2/8-restricted responses of CD4+ T cells to cereal gluten proteins. A diagnosis of celiac disease based on serologic and histologic evidence and duodenal histology requires patients to be on gluten-containing diets. The growing number of individuals adhering to a gluten-free diet (GFD) without exclusion of celiac disease complicates its detection. HLA-DQ–gluten tetramers can be used to detect gluten-specific T cells in blood of patients with celiac disease, even if they are on a GFD. We investigated whether an HLA-DQ–gluten tetramer-based assay accurately identifies patients with celiac disease.

**Methods:** We produced HLA-DQ–gluten tetramers and added them to peripheral blood mononuclear cells isolated from 143 HLA-DQ2.5+ subjects (62 subjects with celiac disease on a GFD, 19 subjects without celiac disease on a GFD [due to self-reported gluten-sensitivity], 10 subjects with celiac disease on a gluten-containing diet, and 52 presumed healthy individuals [controls]). T cells that bound HLA-DQ–gluten tetramers were quantified by flow cytometry. Laboratory tests and flow cytometry gating analyses were performed by researchers blinded to sample type, except for samples from subjects with celiac disease on a gluten-containing diet. Test precision analyses were performed using samples from 10 subjects.

**Results:** For the HLA-DQ–gluten tetramer-based assay, we combined flow-cytometry variables in a multiple regression model that identified individuals with celiac disease on a GFD with an area under the receiver operating characteristic curve (AUROC) value of 0.96 (95% CI, 0.89–1.00) vs subjects without celiac disease on a GFD. The assay detected individuals with celiac disease on a gluten-containing diet vs controls with an AUROC value of 0.95 (95% CI, 0.90–1.00). Optimized cut-off values identified subjects with celiac disease on a GFD with 97% sensitivity (95% CI, 0.92–1.00) and 95% specificity (95% CI, 0.84–1.00),

vs subjects without celiac disease on a GFD. The values identified subjects with celiac disease on a gluten-containing diet with 100% sensitivity (95% CI, 1.00–1.00] and 90% specificity (95% CI, 0.83–0.98) vs controls. In an analysis of 4 controls with positive results from the HLA-DQ–gluten tetramer test, 2 had unrecognized celiac disease and the remaining 2 had T cells that proliferated in response to gluten antigen in vitro.

**Conclusions:** An HLA-DQ–gluten tetramer-based assays that detects gluten-reactive T cells identifies patients with and without celiac disease with a high level of accuracy, regardless of whether the individuals are on a GFD. This test would allow individuals with suspected celiac disease to avoid gluten challenge and duodenal biopsy, but requires validation in a larger study. Clinicaltrials.gov no: NCT02442219

**KEY WORDS:** CD38, gluten-sensitive, gut-homing, non-invasive test

## Introduction

Celiac disease is a gluten-induced enteropathy treated with a life-long gluten-free diet (GFD).<sup>1,2</sup> Around 90% of disease cases are HLA-DQ2.5<sup>+</sup>, the remaining are either HLA-DQ8<sup>+</sup> or HLA-DQ2.2<sup>+</sup>.<sup>3</sup> CD4<sup>+</sup> T cells recognizing deamidated gluten peptides in the context of these HLA-DQ molecules are a hallmark of the disease.<sup>4</sup> Celiac disease patients are usually identified by detection of elevated anti-transglutaminase 2 (anti-TG2) IgA and/or anti-deamidated gliadin peptide (anti-DGP) IgG antibodies in serum. In adults and about half of children,<sup>5</sup> the diagnosis is confirmed by demonstrating typical histological changes in duodenal biopsies.<sup>6</sup>

Partly owing to a pronounced increase in the consumption of gluten-free foodstuffs in the general population,<sup>7</sup> many individuals presenting to the clinician are on a self-instituted GFD without prior diagnostic work-up for celiac disease. Much as a diagnostic work-up is recommended in this situation,<sup>1,2</sup> it poses a substantial challenge for clinicians because initiation of a GFD decreases the sensitivity of histology- and antibody-based tests for celiac disease.<sup>8,9</sup> In such cases, a gluten challenge with several weeks' duration followed by gastroduodenoscopy is suggested by the guidelines.<sup>1,2</sup> A thoroughly conducted 6-week gluten challenge study of subjects with celiac disease treated with a GFD eating 3 g and 6 g gluten daily, gave correct diagnostic histology in 71% and 73%, respectively.<sup>10</sup> Increasing the duration of challenge to 12 weeks (three to five grams gluten daily) did not further increase the sensitivity.<sup>11</sup> Apart from the clinical challenges related to the low sensitivity of the gluten challenge protocol, some patients may not adhere to the protocol if having experienced gluten-related symptoms in the past. Thus, improved diagnostic tests for this patient group are much needed.

Over the last decade, we and others have characterized the diagnostic potential of gluten-specific T cells detected in blood after a short gluten challenge.<sup>12-16</sup> Moreover, by using HLA-DQ–gluten tetramers, we recently demonstrated that gluten-specific T cells are detectable in

blood of both untreated and treated celiac disease patients with a higher frequency than in non-celiac subjects, even in the absence of a gluten challenge.<sup>17</sup> In the present study we investigated the sensitivity and specificity of a modified version of this protocol for detection of celiac disease, in gluten-free and gluten consuming subjects separately. We evaluated this approach in the work-up of celiac disease as an alternative to gluten challenge in gluten-free subjects. Moreover, we explored its potential as a supplement to duodenal biopsy and serology in the work-up of subjects on a normal diet.

## Materials and Methods

### *Trial design, inclusion and recruitment:*

We included HLA-DQ2.5<sup>+</sup>-adults in two groups with gluten-free subjects; 62 subjects with celiac disease treated with a GFD (TCD) and 19 subjects without celiac disease on a GFD due to self-reported gluten sensitivity (GS), and two groups with gluten consuming subjects; 10 untreated subjects with celiac disease on a gluten-containing diet (UCD)-subjects and 52 presumed healthy individuals on a gluten-containing diet (controls) (Supplementary figure 1). Subjects were included in the TCD-group only if the celiac disease diagnosis was based on a duodenal biopsy and if they had been compliant with a GFD since (minimum requirement Biagi score  $\geq 1$ ; transgression of GFD limited to “just a taste” in rare events).<sup>18</sup> Similarly, GS-subjects were included only if celiac disease was excluded by duodenal histology while on a gluten-containing diet for at least three weeks and if they reported adequate compliance (Biagi score  $\geq 1$ ). We did not confirm self-reported gluten sensitivity by food challenge. Controls denied any prior history of celiac disease and had been on a normal gluten-containing diet for at least 6 months. Exclusion criteria were immune suppressive treatment for the last 3 months, pregnancy, hepatitis B or C and a positive HIV status.

We recruited subjects by invitation and by announcements at the hospital website, celiac disease patient organization website, nearby clinics and via social media from May 2015 to February 2016. UCD-subjects were included consecutively amongst seropositive patients referred to our endoscopy unit at Oslo University Hospital from November 2015 to June 2016 as part of their regular secondary line care (the majority of endoscopies are done at outpatient clinics in our public health care system). If not already done, potential participants were HLA-typed (LABType™ SSO, ONE LAMBDA, Los Angeles, CA).

In addition, we examined analytical and weekly variation of the HLA-DQ–gluten tetramer test. For this purpose, we included five TCD-subjects and five controls selected from the primary study cohort to represent different degrees of test response. Analytical variation was determined by comparing two halves of a blood sample divided prior to processing and biological variation by comparing two samples drawn one week apart from the same subject on an unaltered diet.

*Sampling, blinding protocol and data collection:*

We drew 54 mL citrated blood for the HLA-DQ–gluten tetramer test. Subjects met for blood sampling after randomization, the samples were de-identified (except for UCD-group) and processed the following day for flow cytometry. Flow-cytometric analysis, including the gating of cell populations, was done blinded for subject identity. Additional serum for anti-TG2 IgA (reference range < 3 units/mL, VarElisa Celikey IgA, Phadia, Freiburg, Germany), anti-DGP IgG (reference range < 20 units, QUANTA Lite™ Gliadin IgG II, INOVA Diagnostics Inc., San Diego, CA), total IgA and C-reactive protein was collected for later analysis. Gastrointestinal symptoms were scored by a self-administered questionnaire (Gastrointestinal Symptoms Rating Scale – irritable bowel syndrome version) on the day of blood sampling.<sup>19</sup>

*HLA-DQ–gluten tetramers and flow cytometry analysis:*

The HLA-DQ–gluten tetramers were produced as previously described.<sup>20</sup> Recombinant HLA-DQ2.5 (DQA1\*05:01/ DQB1\*02:01) molecules presenting the epitope peptides HLA-DQ2.5-

glia- $\alpha$ 1a (QLQPFQPELPY, with underlined 9-mer core sequence), DQ2.5-glia- $\alpha$ 2 (PQPELPYPQPE), DQ2.5-glia- $\omega$ 1 (QQPFQPEQPFQ), DQ2.5-glia- $\omega$ 2 (FPQPEQFPWQP) and DQ2.5-hor-3 (PIPEQPYPYQ) were produced in a baculovirus expression system, multimerized on phycoerythrin-labeled streptavidin, and mixed together to 10  $\mu$ g/ml each before incubation with peripheral blood mononuclear cells (PBMC). Anti-phycoerythrin microbeads and magnetic columns (autoMACS<sup>®</sup> Pro Separator, Milenyi Biotec, Bergisch Gladbach, Germany) were used to enrich HLA-DQ–gluten tetramer-binding cells prior to staining with a mixture of monoclonal antibodies; CD45RA-PE-Cy7, CD3-eVolve 605 and CD38-FITC (eBioscience, Thermo Fisher Scientific, Waltham, MA), CD11c-PB and CD4-APC-H7 (BD Biosciences, San Jose, CA) and CD62L-PerCP/Cy5.5, integrin  $\beta$ 7-APC, CD14-PB, CD19-PB, CD56-PB (BioLegend, San Diego, CA). We defined the two test-relevant cell populations, HLA-DQ–gluten tetramer<sup>+</sup> and HLA-DQ–gluten tetramer<sup>-</sup> cells, by flow-cytometric gating analysis as cells that were single-cell lymphocytes (defined by forward-scatter and side-scatter), CD3<sup>+</sup>CD11c<sup>-</sup>CD14<sup>-</sup>CD19<sup>-</sup>CD56<sup>-</sup>CD4<sup>+</sup> and HLA-DQ–gluten-tetramer-binding positive and negative respectively (Figure 1A). Both test-relevant cell populations were further gated for CD45RA<sup>+</sup>CD62L<sup>+</sup> naïve T-cells (T<sub>N</sub>) and CD45RA<sup>-</sup>CD62L<sup>-</sup> effector memory T-cells (T<sub>EM</sub>). T<sub>EM</sub> were also gated for integrin- $\beta$ 7<sup>+</sup> ( $\beta$ 7<sup>+</sup>) gut homing. The number of gluten-specific T cells (HLA-DQ–gluten-tetramer<sup>+</sup> $\beta$ 7<sup>+</sup>T<sub>EM</sub>) was divided by the total number of CD4<sup>+</sup> T cells in the sample to calculate the frequency. The total number of CD4<sup>+</sup> T cells was estimated by taking a separate sample before enrichment with HLA-DQ–gluten-tetramers to establish the CD4<sup>+</sup>-ratio in PBMC and multiply it with the total PBMC-count (Figure 1B).

#### *HLA-DQ–gluten tetramer test variables*

We used flow-cytometric raw-data to calculate pre-defined parameters (frequency of gluten-specific T cells and variables 1 – 3) by dividing the cell-population in question with a suitable

reference cell-population in the same sample to correct for sample- and subject-dependent background variability and thereby to optimize normalization of variables (Table 1).

### *Data transformations and multiple regression analysis*

The variables were Box-Cox power transformed [B-C; $\lambda$ ] to correct for skewness.<sup>21, 22</sup> A common optimal lambda ( $\lambda$ ) was established for GS and TCD groups; as well as for control and UCD groups. Stepwise multivariate linear regression analyses on transformed variables were done for the comparisons GS vs TCD and control vs UCD to find the optimal models. Four seropositive controls (anti-TG2 IgG > 3 units/mL or anti-DGP IgG > 20 units) were excluded from the regression analyses, but not excluded from calculation of diagnostic accuracy.

Data values in variables 2 - 3 were considered unreliable if  $T_{EM} \leq 5$  (or if  $T_N \leq 5$  for variable 3), and for those cases the average value of the non-celiac group was substituted. For variable 1,  $T_N$  was set to 1 if no such cells were detected.  $CD38_{RR}$ , a parameter for CD38-expression, was only evaluated in cases where the number of gluten-specific T-cells was greater than five.

### *Cell-culture and proliferation assay*

T-cell lines were generated from bulk-sorted HLA-DQ–gluten-tetramer<sup>+</sup> $\beta 7^+$  $T_{EM}$  by antigen free stimulation as previously described.<sup>23</sup> Antigen-dependent T-cell proliferation assay was performed using a well-established protocol by using HLA-DQ2.5<sup>+</sup> homozygous Epstein-Barr virus (EBV)-transformed cells (IHW #9023) for presentation of the gluten peptide-antigens (final concentration 10  $\mu$ M) assessed by <sup>3</sup>H-thymidine incorporation. T cells were considered specific for an antigenic peptide if the stimulation index was above three, meaning that the peptide stimulation resulted in a measured radioactivity at least three times that observed without peptide.<sup>23</sup>

### *Statistics*

Box-Cox data transformations were done in Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA) and multiple regression analyses by SPSS (IBM SPSS Statistics V22.0, North Castle, NY). Receiver operating characteristic (ROC) analysis and significance tests were performed using GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA). Optimal cut-off values in the ROC analyses were defined by the value lying closest to the coordinate point (0,1).<sup>24</sup> Significance tests were performed with Fisher's exact test in the case of dichotomous variables. For continuous variables, we performed two sample t-tests when data had an approximately normal distribution, and Mann-Whitney test elsewhere. Confidence intervals for accuracy estimates were calculated by bootstrapping. Data points with values below the lower detection limit were assigned half its value. The significance level was  $P < .05$ .

#### *Ethical aspects*

The study was approved by the regional ethical committee of South-East Norway (REK accession numbers 2010/2720 and 2011/2472) and registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT02442219). Written informed consent was obtained from all participants. All authors had access to the study data and reviewed and approved the final manuscript.

## Results

### *Participant characteristics*

Both non-celiac groups (GS and control) were similar regarding age, body mass index, first degree relatives with celiac disease, daily smoking, other auto-immune disease, gastrointestinal symptoms and serum C-reactive protein level (Supplementary Table 1). The gluten-free TCD and GS groups were also similar in all these aspects in addition to adherence to GFD, but differed in duration of GFD ( $P = .034$ ). The GS-group of 19 subjects was relatively small due to the strict inclusion-criteria requiring previous exclusion of celiac disease by duodenal histology while these subjects were still consuming gluten. Partly due to issues of recruitment, we limited the size of the UCD-group to 10 subjects, also because we expected larger differences in test response between celiac and non-celiac subjects in the groups with gluten consuming subjects than in the groups with gluten-free subjects.

### *Antibody-based tests were not sensitive for detecting TCD subjects*

Of the 10 UCD-subjects, nine were anti-TG2 IgA-positive and eight were anti-DGP IgG-positive, significantly different from the antibody positivity in TCD and control groups ( $P < .001$  for UCD vs TCD and UCD vs control for both antibodies) (Figure 2 & Supplementary Table 1). One of 62 TCD-subjects was positive for anti-TG2 IgA only, four subjects were positive for anti-DGP IgG only, and one had elevated levels for both. Notably, four of 52 controls were seropositive for anti-DGP IgG and only these four subjects had measurable anti-TG2 IgA levels ( $\geq 1$  unit/mL) amongst controls. All GS-subjects had undetectable anti-TG2 IgA levels, and all but one had undetectable anti-DGP IgG levels. No subjects had IgA deficiency.

### *Flow-cytometric variables were significantly different in non-celiac and celiac disease subjects*

We assessed the ability of four variables based on flow-cytometric cell surface markers (Table 1) to correctly classify the study participants as having celiac disease. The variables represent ratios of different flow-cytometric parameters between the HLA-DQ–gluten tetramer-binding cells vs non-binding cells. The use of ratios partially compensates for sample-to-sample variations in terms of gating strategy, counting errors and sample handling. In short, variable 1 represents the ratio of effector memory vs naive T cells, variable 2 the integrin  $\beta 7$  expression on effector memory cells and variable 3 the staining intensity for HLA-DQ–gluten tetramer. Variables 1 – 3 and frequency of gluten-specific T cells were all significantly different when comparing TCD vs GS ( $P < .001$ ) (Figure 3 & Supplementary Table 1). The difference between groups was also significant for the comparison UCD vs control ( $P \leq .001$ ) for all variables, except variable 3.

*Optimal multiple regression models accurately differentiate non-celiac from celiac disease subjects, regardless of gluten consumption*

For more accurate classification of subjects, we applied multiple regression analysis to construct two models based on variables 1 – 3 (Figure 4A & 4C). We developed one model for the comparison TCD vs GS using all three variables as significant contributors, and the second model for UCD vs control with variables 1 – 2 as significant contributors (Table 2). The parameter for frequency of gluten-specific T cells was strongly correlated to variable 1 for both comparisons (Pearson correlation  $r = 0.84$  for GS vs TCD and  $r = 0.76$  for control vs UCD after data transformation). This parameter was therefore omitted from the regression analysis for the benefit of variable 1, a variable that did not require additional counting and staining of a pre-tetramer-enriched sample.

ROC analysis defined the optimal cut-offs, giving an AUROC value of 0.96 (95% CI, 0.89–1.00) for TCD vs GS and 0.95 (95% CI, 0.90–1.00) for UCD vs control (Figure 4B & 4D). The corresponding sensitivity and specificity for the TCD vs GS groups were 0.97 (95% CI, 0.92–

1.00) and 0.95 (95% CI, 0.84–1.00), and for the UCD vs control groups 1.00 (95% CI, 1.00–1.00) and 0.90 (95% CI, 0.83–0.98), respectively.

Notably, four of the five controls who tested positive on the index test (i.e. the HLA-DQ–gluten tetramer test) (Figure 4C), were found to be seropositive for celiac disease specific antibodies. One subject in the GS-group was index test positive. Although this GS participant documented a previously negative duodenal biopsy at the time of inclusion, when confronted with a positive index test result, she recalled that the biopsy was done after only two weeks of gluten challenge and a strict GFD for several months prior to that. We rechecked the biopsy and verified normal histology. Two subjects in the TCD-group were index test negative. One of them, diagnosed as a child in the early 1970's, participated also in a two-week gluten challenge study and remained both histology and antibody negative.<sup>25</sup>

*In controls with high frequency of HLA-DQ–gluten tetramer<sup>+</sup> cells, these cells were specific to gluten upon stimulation in vitro*

Four of five index test positive controls, who were found to be seropositive for either anti-TG2 IgA or anti-DGP IgG, accepted invitation for diagnostic work-up of celiac disease in addition to two index-test negative and seronegative controls with a frequency of gluten-specific T cells on same level as UCD-subjects. All six control subjects underwent duodenal biopsies and celiac disease specific serology, 6 – 12 months after the index test. Two index test positive controls had villous blunting on histological evaluation, and thus diagnosed with celiac disease (Supplementary Table 2). Among the remaining four individuals, the two index test positive subjects had some, but not sufficient histological changes to qualify for celiac disease diagnosis, and the two index test negative subjects had completely normal duodenal histology (Supplementary Table 2). In these four non-celiac controls, flow cytometry of PBMC 6 – 12 months after the initial test showed an unaltered high frequency of HLA-DQ–gluten tetramer-binding cells (Supplementary Figure 2A). To confirm gluten reactivity, we sorted HLA-DQ–gluten-tetramer<sup>+</sup>β7<sup>+</sup>T<sub>EM</sub> from these control subjects and cultured the cells as T-cell

lines in absence of antigenic stimulation. Subsequently, the T-cell lines were stimulated with native gluten, deamidated gluten (TG2-treated) and with the five gluten-epitopes that were represented during HLA-DQ–gluten tetramer cell sorting. We found positive proliferative responses in all T-cell lines towards deamidated gluten and towards most of the peptides (Supplementary Figure 2B).

*CD38-expression on gluten-specific T cells is as accurate as antibody-based tests to differentiate TCD from UCD*

Motivated by previous results on CD38 as a marker that was upregulated on gluten-specific T cells after gluten challenge,<sup>26</sup> we investigated whether CD38-expression on HLA-DQ–gluten-tetramer<sup>+</sup>β7<sup>+</sup>T<sub>EM</sub> was associated with the treatment-status of the celiac disease patients. The frequency of CD38<sup>+</sup> gluten-specific T-cells was low in the non-celiac groups except for six controls with similar levels as the UCD-subjects, where the four seropositive controls displayed the topmost frequency (Supplementary Figure 3A). We explored the celiac disease groups in greater detail by using the flow-cytometric variable CD38<sub>RR</sub>, an estimate of CD38-expression normalized for background subject variability (Table 1), and found the groups (TCD vs UCD) to be significantly different ( $P < .001$ ) (Supplementary Figure 3B). Further, ROC analysis for prediction of treatment status (UCD vs TCD) resulted in an AUROC value of 0.96 (95% CI, 0.91–1.00), and an optimized cut-off gave sensitivity 0.90 (95% CI, 0.70–1.00) and specificity 0.93 (95% CI, 0.87–0.98) (Supplementary Figure 3C). By comparison, sensitivity and specificity were 0.90 (95% CI, 0.70–1.00) and 0.97 (95% CI, 0.92–1.00) for anti-TG2 IgA, and 0.80 (95% CI, 0.50–1.00) and 0.92 (95% CI, 0.84–0.98) for anti-DGP IgG, respectively, for differentiating between UCD and TCD.

*Analytical variation of the HLA-DQ–gluten tetramer test equals the weekly biological variation*

The test of precision performed on samples from five TCD subjects and five controls that were divided into two halves before processing (analytical variation), showed a variation

approximately equal to that of samples taken with one week interval (Supplementary Figure 4 and Supplementary Table 3).

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## Discussion

In this study of 143 participants we demonstrate that the HLA-DQ–gluten tetramer blood test is sensitive and specific for celiac disease. Unlike established tests, this test does not require gluten challenge for subjects on a GFD for assessment of disease status (sensitivity 0.97 [95% CI, 0.92–1.00] and specificity 0.95 [95% CI, 0.84–1.00]). The test will allow exclusion of celiac disease for a large proportion of subjects on a GFD without a diagnosis. For subjects who are on a normal gluten-containing diet, sensitivity (1.00 [95% CI, 1.00–1.00]) and specificity (0.90 [95% CI, 0.83–0.98]) of the HLA-DQ–gluten tetramer test was comparable to the accuracy of celiac disease specific antibody tests, thus providing a new and less invasive supplement to existing tests.

CD is highly associated with certain HLA-allotypes,<sup>3</sup> and HLA-typing is utilized in clinical work-up of celiac disease.<sup>1, 2</sup> Although the absence of these disease-associated HLA allotypes may exclude celiac disease, the positive predictive value of the HLA-DQ2/8-typing remains low.<sup>3</sup> Determining the presence of gluten-reactive T cells in patients who carry celiac disease associated HLA-DQ allotypes may assist in further differentiation of disease status. The CD4<sup>+</sup> T-cell reactivity in relation to celiac disease has been assessed by either HLA-DQ–gluten tetramer analysis or by T-cell cytokine release assays (ELISPOT / ELISA).<sup>13, 15-17, 25, 27-29</sup> The HLA-DQ–gluten tetramer approach uses fluorescent complexes of gluten peptides tethered to disease-specific HLA molecules for direct detection of gluten-specific cells in PBMC by flow cytometry.<sup>20</sup> In the ELISPOT test, PBMC are incubated with gluten-epitopes overnight, followed by an interferon- $\gamma$  ELISPOT assay.<sup>27</sup> In the ELISA test, measurement of interferon- $\gamma$  or interferon- $\gamma$ -inducible protein 10 is done in whole blood after an overnight incubation with gluten-peptides.<sup>16</sup> With both ELISPOT and ELISA, the T-cell reactivity in PBMC is detectable only when combined with a 3-day oral gluten challenge and blood-sampling on day 6,<sup>12, 16, 28, 29</sup> when there is a surge of gluten-reactive T-cells in the blood.<sup>27</sup> Initially, the HLA-DQ–gluten tetramer approach also was used to detect a T-cell response to gluten in PBMC on day 6 after a 3-day oral gluten challenge.<sup>13, 15</sup> In a later study, the

sensitivity of the assay was increased by magnetic bead enrichment for tetramer-binding T cells,<sup>30</sup> as positive response was detected in 11 of 13 HLA-DQ2.5<sup>+</sup> TCD-subjects without the need of a gluten challenge.<sup>17</sup> In the current study, we further improved the protocol for the tetramer-based approach by pooling together five HLA-DQ–gluten tetramers, implementing gut-homing staining to all samples, reducing the required blood volume for the assay to an acceptable level and applying protocol automation for magnetic bead enrichment. Moreover, we identified and integrated new flow-cytometric variables to generate models that demonstrated a high degree of accuracy for correct prediction of celiac disease in a larger number of gluten consuming and gluten-free subjects, without applying gluten challenge.

The reference standard in the gluten-free groups of this study was exclusion of celiac disease by duodenal biopsy while the subjects were still consuming gluten. However, one index test positive GS-subject had inadequate prior exclusion of celiac disease, and one index test negative TCD-subject, diagnosed with celiac disease in the early 1970's, did not respond with duodenal changes after a two-week gluten challenge in another related study.<sup>25</sup> Both subjects may thus have contributed to a lower than actual estimate of sensitivity and specificity. By comparison, the highest reported estimates of diagnostic sensitivity of 2 – 12-week gluten challenge followed by duodenal biopsy are approximately 70%.<sup>10, 11, 31</sup> This sensitivity may also be much lower, depending on the dose and duration of gluten challenge.<sup>25</sup> Thus, introducing the HLA-DQ–gluten tetramer blood test in this clinical setting could clearly benefit patients and clinicians by offering a faster, more sensitive and symptom-free test, as gluten challenge is known to induce unacceptable symptoms in some patients. The new test may thus potentially replace gluten challenge followed by duodenal biopsy. However, as a result of the low prevalence of celiac disease among subjects on a self-instituted GFD, estimated to be about 10% in an HLA-DQ2.5<sup>+</sup> population in our region,<sup>15</sup> the estimated positive predictive value of the HLA-DQ–gluten tetramer test would be 0.67, whereas the negative predictive value would be 1.00. Due to the superior negative predictive value, we recommend a strategy to use the new test for exclusion of celiac disease. To be

compliant with current guidelines for celiac disease diagnosis, we advise that the few subjects who score positive with the HLA-DQ–gluten tetramer test should undergo gluten challenge followed by duodenal biopsy to establish the diagnosis. Hopefully, in the future the performance of the HLA-DQ–gluten tetramer test can be further improved allowing the diagnosis to be made directly without the need for an oral gluten challenge.

The controls were not subjected to duodenal biopsy before inclusion, and some unrecognized cases of celiac disease could therefore be expected. Indeed, four of five controls that tested positive, were also positive for celiac disease specific serology, and two of these seropositive subjects were later diagnosed with celiac disease based on duodenal histology. A prevalence of unrecognized celiac disease of 4% (95% CI, 0–10%) in our cohort of 52 HLA-DQ2.5<sup>+</sup> controls was similar to 3.5% prevalence (95% CI, 2.1%–4.9%) of serology-detected biopsy-proven unrecognized celiac disease found in 655 HLA-DQ2.5<sup>+</sup> Finnish children.<sup>32</sup> Although the prevalence in our cohort was similar to the Finnish cohort, we cannot exclude some degree of self-selection of participants with previous symptoms or signs suggestive of celiac disease in our study. Thus, also in this diagnostic model, rigorous inclusion criteria requiring duodenal histology for all could have resulted in higher test specificity. Nevertheless, the clinical significance of partially or completely negative histology but positive HLA-DQ–gluten tetramer test, indicating an ongoing systemic immune response, should be evaluated further. For instance, the use of T-cell based assays may help to classify the debated condition of potential celiac disease, defined as positive anti-TG2 IgA levels but negative histology,<sup>33-35</sup> as either a pre-stage or a sub-group of celiac disease. If a positive HLA-DQ–gluten tetramer-test, used together with celiac disease specific serology, displays predictive ability for development of celiac disease, it may signify a window of opportunity for early therapeutic intervention.

Serology can detect dietary transgressions in celiac disease patients,<sup>36</sup> and may be used to assess dietary compliance in patients with lack of symptomatic remission after initiation of treatment.<sup>37</sup> Using CD38-expression (CD38<sub>RR</sub>) as a parameter for activation of the HLA–DQ–

gluten-tetramer<sup>+</sup> T cells, we could differentiate TCD-subjects from UCD-subjects with test accuracy similar to serology. CD38 is up-regulated on circulating gluten-specific T cells after a gluten challenge,<sup>26</sup> and has faster kinetics than serology (six days vs weeks to months after initiation of gluten consumption).<sup>10, 25, 28, 38</sup> If these fast kinetics also apply with the termination of gluten intake (currently being evaluated by us in a separate study), CD38<sub>RR</sub> may be used as a faster response parameter in the follow-up of celiac disease. Additionally, it may be used to stratify subjects with reduced gluten intake for the GS–TCD model or control–UCD model of the HLA-DQ–gluten tetramer test.

The suggested diagnostic models should be validated in independent studies. In the case of gluten-free subjects, a validation should be carried out as a multi-center study with a retrospective confirmation of diagnosis as in the current study, or with a prospective design where at risk gluten-free subjects undergo a gluten-challenge as part of routine work-up for celiac disease. Although mimicking the clinical situation, drawbacks with the latter approach could be potential dropouts and a significant proportion of false negative histology outcome, which would complicate calculation of the accuracy of the index test. The study should be done in a blinded manner. In the current study, blinded pre-planned inclusion of UCD-subjects was not feasible, as sampling of blood for the HLA-DQ–gluten tetramer-test had to be done on the day of appointment for the routine gastroduodenoscopic examination due to subsequent initiation of a GFD.

A practical limitation, which may affect children, is the required volume of blood (54 mL). This volume may be reduced, especially if the test subject is consuming gluten. Other limitations may be the requirement to analyze the sample on the same or following day of blood draw, and the duration of time required for blood analysis. The duration of the current protocol was seven hours for five samples, including centrifugation time, incubation periods, automated HLA-DQ–gluten tetramer-enrichment and flow cytometry analysis. Parts of the process may, however, be further automated and protocol modifications for freezing down PBMC from fresh blood may allow for flexibility and increased efficiency. Finally, our study-population

was limited to HLA-DQ2.5<sup>+</sup> subjects. Although, the HLA-DQ–gluten tetramer test may also be feasible in subjects with the other celiac disease associated HLA-types (HLA-DQ8 and HLA-DQ2.2),<sup>25, 39</sup> the diagnostic potential of the HLA-DQ–gluten tetramer test in non-HLA-DQ2.5<sup>+</sup> subjects remains to be tested in studies of adequate size.

In conclusion, the HLA-DQ–gluten tetramer blood test for detection of gluten-specific CD4<sup>+</sup> T cells is a less invasive test for celiac disease and has high sensitivity and specificity, even if the subject is on a GFD. Due to the high negative predictive value in our HLA-DQ2.5<sup>+</sup> patient population of self-instituted GFD, it can replace gluten challenge followed by duodenal biopsy for exclusion of celiac disease in a sizable majority of these patients. The new test should thus provide an attractive option for the many self-diagnosed gluten intolerant subjects, carrying the celiac disease risk gene HLA-DQ2.5 and avoiding dietary gluten. Even though the minority of subjects who are expected to test positive for the new test will be recommended to undergo a gluten challenge and duodenal biopsy to obtain the diagnosis according to current guidelines, finding motivation for the challenge should be easier knowing the high likelihood of a positive diagnostic outcome. The test should also help the clinicians to meet expectations of further risk-stratification beyond HLA-typing of this group of patients who they currently have little to offer but a general recommendation of a gluten challenge. Efforts should be made to validate these test performances and to make the HLA-DQ–gluten tetramer test broadly available.

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Author names in bold designate shared co-first authorship.

#### Figure Legends:

Figure 1. **Flow-cytometric gating strategy.** (A) Gating of the HLA-DQ–gluten tetramer-enriched sample, exemplified in an untreated celiac disease patient (ID 1395). CD4<sup>+</sup> HLA-DQ–gluten tetramer positive and negative fractions were divided into effector memory (CD45RA<sup>+</sup>CD62L<sup>-</sup>) and naïve cells (CD45RA<sup>+</sup>CD62L<sup>+</sup>). Effector memory cells were analyzed for integrin  $\beta$ 7-expression and integrin  $\beta$ 7<sup>+</sup> cells were further gated for CD38-expression. CD4<sup>+</sup> HLA-DQ–gluten tetramer-negative cells were used as a reference for gating and as a means of variable normalization for the HLA-DQ–gluten tetramer-positive population. (B) The gating strategy of the sample prior to HLA-DQ–gluten tetramer-enrichment (pre-sample). The pre-sample was used to establish the ratio of CD3<sup>+</sup>CD4<sup>+</sup> cells within PBMC.

Figure 2. **Serum antibody levels.** (A) Values of IgA anti-transglutaminase 2 (anti-TG2) and (B) IgG anti-deamidated gliadin peptide (anti-DGP) antibodies are depicted for all participants in the four groups. Black dotted lines denote the upper reference levels (3 units/ml for anti-TG2 and 20 units for anti-DGP). For the purpose of visualization, data points below the lower limit of detection (stippled lines, IgA anti-TG2 = 1, IgG anti-DGP = 5) were given half the value of the lower limit of detection. *n* indicates the number of subjects in each group. GS, self-reported gluten sensitive; TCD, treated celiac disease; UCD, untreated celiac disease.

Figure 3. **Flow-cytometric variables applied for differentiation of groups.** (A) Frequency of gluten-specific T cells, HLA-DQ–gluten-tetramer<sup>+</sup>β7<sup>+</sup>T<sub>EM</sub> / 10<sup>6</sup> CD4<sup>+</sup> T cells. (B) Ratio of T<sub>EM</sub> to T<sub>N</sub> in HLA-DQ–gluten tetramer<sup>+</sup> cells divided by the same ratio in the HLA-DQ–gluten tetramer<sup>-</sup> cells (variable 1). (C) Ratio of β7<sup>+</sup> in HLA-DQ–gluten-tetramer<sup>+</sup>T<sub>EM</sub> divided by the same ratio in the HLA-DQ–gluten-tetramer<sup>-</sup>T<sub>EM</sub> (variable 2). (D) Median HLA-DQ–gluten tetramer staining-intensity in HLA-DQ–gluten-tetramer<sup>+</sup>T<sub>EM</sub> relative to staining-intensity in HLA-DQ–gluten-tetramer<sup>+</sup>T<sub>N</sub> (variable 3). *n* indicates the number of subjects in each group with valid data for the variable. Open circles in the GS and control groups represent participants with an elevated antibody titer (anti-TG2 > 3 units/ml or anti-DGP > 20 units). Values equal to 0 are set to 0.01 on the logarithmic axis for the purpose of visualization.

Figure 4. **Multiple regression models for groups with gluten-free and gluten consuming subjects with optimal cut-offs.** (A and C) Models were established after power transformation and multiple regression analysis of variables 1 - 3. (B and D) Receiver operating characteristics (ROC) curves are shown for each of the models in the following panels. (A) TCD-subjects were compared with GS-subjects and (B) the associated ROC curve show cut-off for optimal sensitivity and specificity (i.e. the ROC-curve value closest to coordinate point (0,1)). The same analyzes were done for UCD-subjects vs controls. Open circles in the GS and control groups represent participants with an elevated antibody titer

(anti-TG2 IgA > 3 units/ml or anti-DGP IgG > 20 units). Solid horizontal lines indicate mean values. Dotted lines in panels with ROC curves (B and D) show the optimal cut-offs and dotted horizontal lines in the associated models (A and C, respectively) indicate these cut-offs as applied. AUROC; area under the ROC curve.

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Tables:

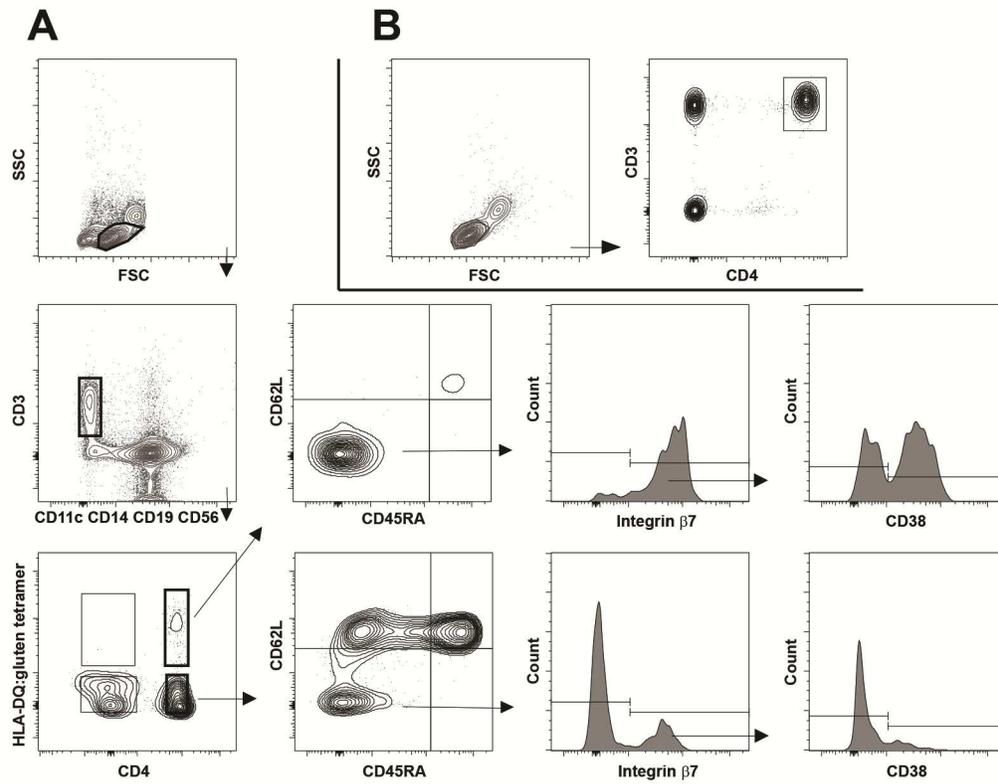
Table 1. **Flow-cytometric variables.**

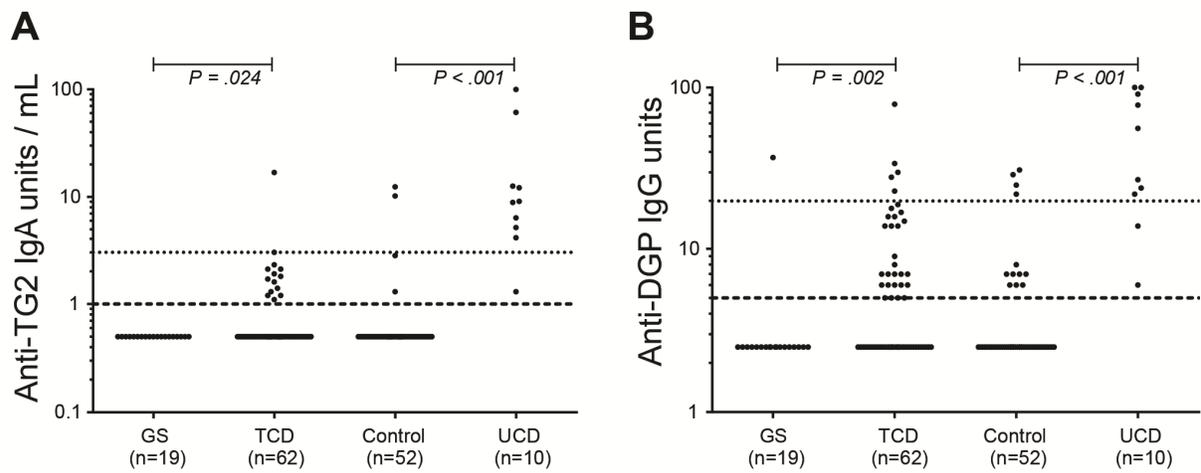
Variable	Numerator	Denominator
Frequency of gluten-specific T cells	HLA-DQ–gluten-tetramer <sup>+</sup> β7 <sup>+</sup> T <sub>EM</sub>	10 <sup>6</sup> CD4 <sup>+</sup> T cells
Variable 1	T <sub>EM</sub> / T <sub>N</sub> in HLA-DQ–gluten-tetramer <sup>+</sup> cells	T <sub>EM</sub> / T <sub>N</sub> in HLA-DQ–gluten-tetramer <sup>-</sup> cells
Variable 2	β7 <sup>+</sup> ratio in HLA-DQ–gluten-tetramer <sup>+</sup> T <sub>EM</sub>	β7 <sup>+</sup> ratio in HLA-DQ–gluten-tetramer <sup>-</sup> T <sub>EM</sub>
Variable 3	Median HLA-DQ–gluten tetramer staining-intensity in HLA-DQ–gluten-tetramer <sup>+</sup> T <sub>EM</sub>	Median HLA-DQ–gluten tetramer staining-intensity in HLA-DQ–gluten-tetramer <sup>+</sup> T <sub>N</sub>
CD38 relative ratio (CD38 <sub>RR</sub> )	CD38 <sup>+</sup> ratio in HLA-DQ–gluten-tetramer <sup>+</sup> β7 <sup>+</sup> T <sub>EM</sub>	CD38 <sup>+</sup> ratio in HLA-DQ–gluten-tetramer <sup>-</sup> β7 <sup>+</sup> T <sub>EM</sub>

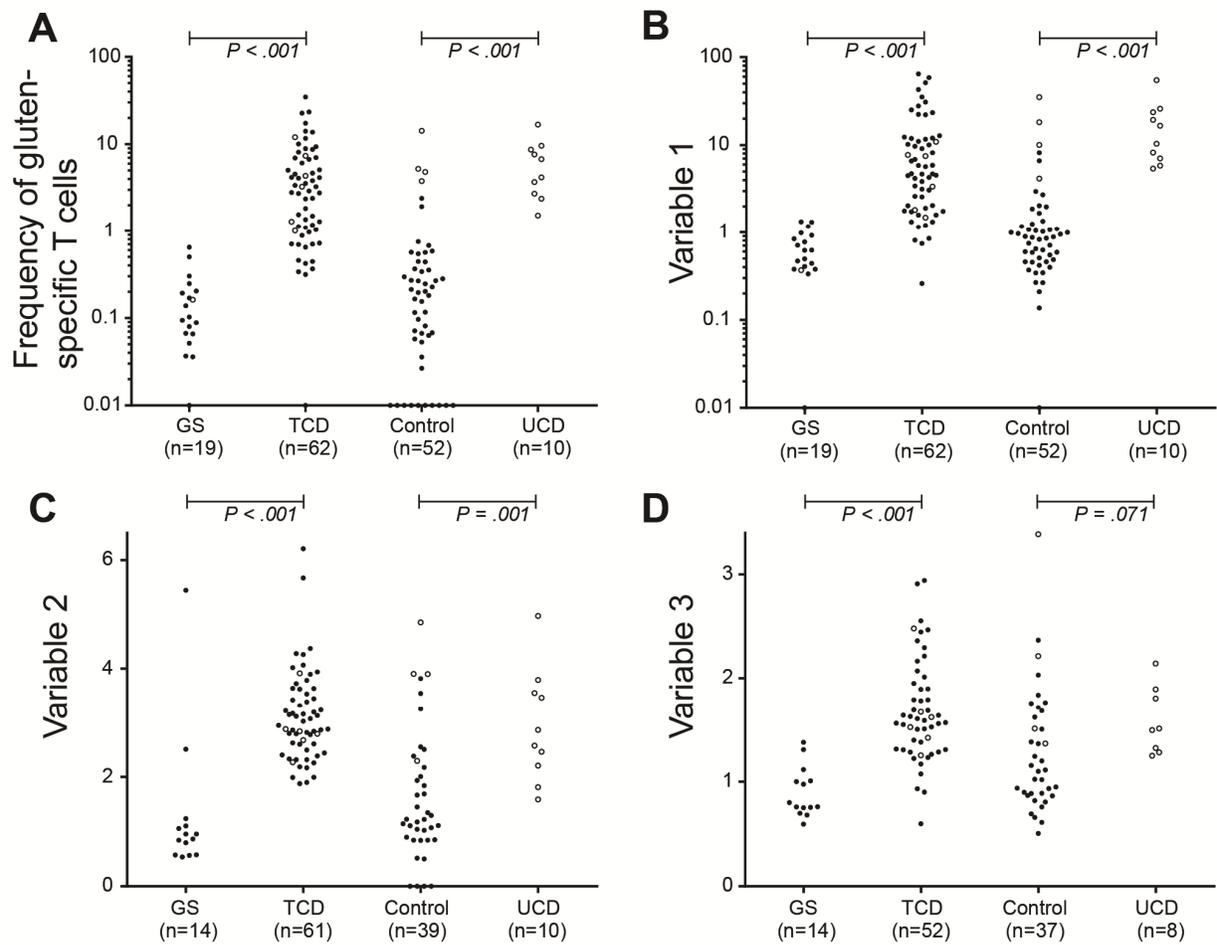
Table 2. **Optimal models for differentiation of non-celiac from celiac disease subjects.**

Comparison	Variables	Substitutions	Transformations	Model	Optimal cut-off
Gluten free: Non-celiac vs celiac subjects ( <b>GS – TCD</b> )	V1	V1 → T <sub>N</sub> = 1 if T <sub>N</sub> = 0	x = [B-C; -0.01] V1	U <sub>i</sub> = 0.28 +0.39· x <sub>i</sub>	0.347
	V2	V2 = 1.287 if T <sub>EM</sub> ≤ 5;	y = [B-C; 0.10] V2	+0.072· x <sub>i</sub>	
	V3	V3 = 0.899 if T <sub>EM</sub> ≤ 5 or T <sub>N</sub> ≤ 5;	z = [B-C; 0.19] V3	+0.19· z <sub>i</sub> + ε <sub>i</sub>	
Gluten consuming: Non-celiac vs celiac subjects ( <b>Control – UCD</b> )	V1	V1 → T <sub>N</sub> = 1 if T <sub>N</sub> = 0	x = [B-C; -0.04] V1	U <sub>i</sub> = 0.069 +0.12· x <sub>i</sub>	0.479
	V2	V2 = 1.635 if T <sub>EM</sub> ≤ 5;	y = [B-C; 0.36] V2	+0.14· y <sub>i</sub> +ε <sub>i</sub>	

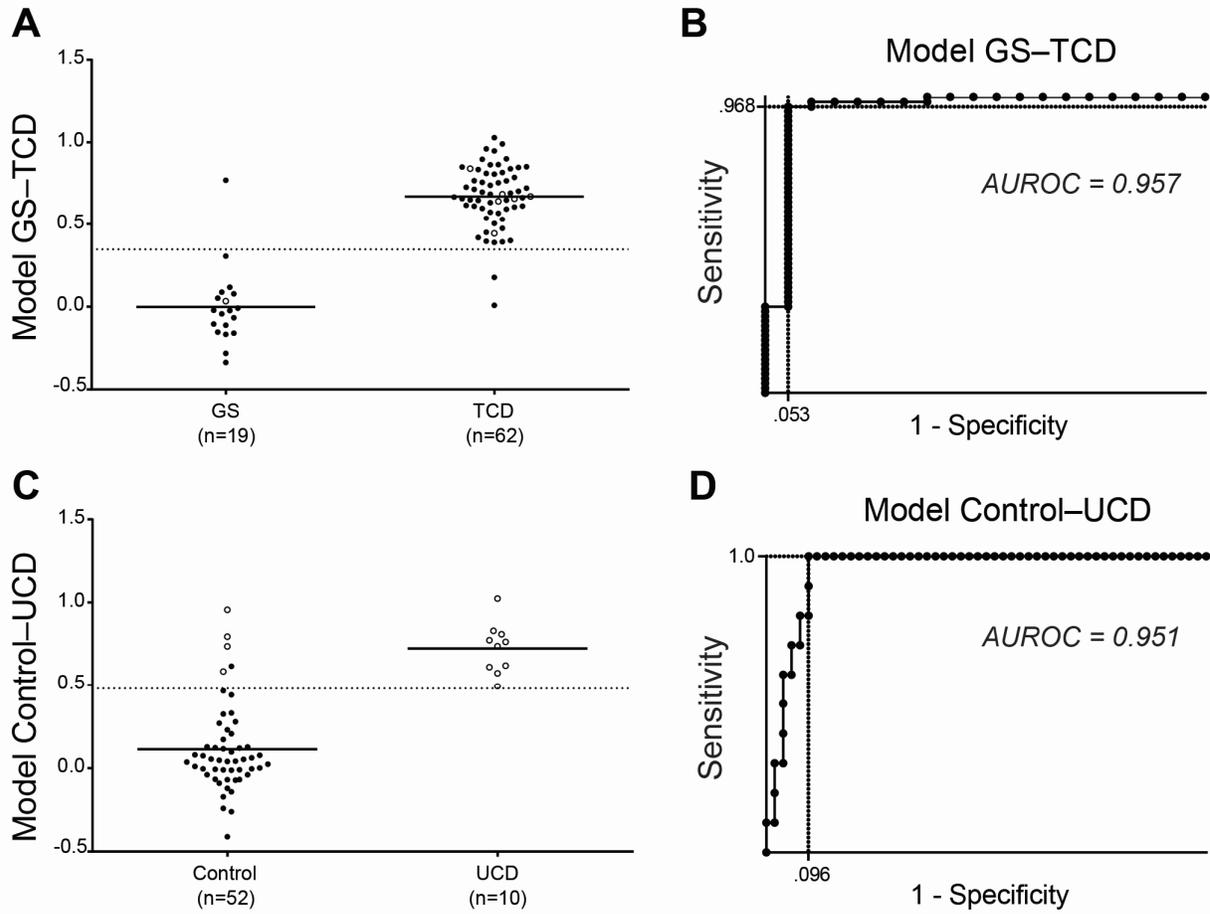
U = 0 for non-celiac group and 1 for celiac disease group. ε denotes residuals; i = 1,2...n; V 1-3, variables 1-3; T<sub>N</sub> and T<sub>EM</sub> denote naïve and effector memory T cells (respectively) in HLA-DQ-tetramer<sup>+</sup> fraction.







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Supplementary table 1. Characterization of participant groups

Variables	Controls	Gluten sensitive (GS)	Treated celiac disease (TCD)	Untreated celiac disease (UCD)	P-value controls vs GS	P-value GS vs TCD	P-value TCD vs UCD	P-value controls vs UCD
<b>Participants; n (male/female)</b>	52 (20/32)	19 (3/16)	62 (10/52)	10 (3/7)				
<b>Age; mean [95% confidence interval]; years</b>	37.9 [35.3 , 40.6]	43.0 [37.0 , 49.0]	43.4 [40.5 , 46.4]	30.8 [20.6 , 41.0]	0.076	0.89	<b>0.003</b>	0.053
<b>BMI; median (25% , 75%); kg/m<sup>2</sup></b>	24.0 (21.3 , 27.3)	24.2 (21.6 , 29.2)	23.9 (22.3 , 26.6)	nd	0.832	0.947		
<b>Duration of GFD; median (25% , 75%), months</b>	NA	24.0 (11.0 , 55.0)	57.5 (19.0 , 118.0)	NA		<b>0.034</b>		
<b>First degree relatives with celiac disease; %</b>	15.4	31.6	41.9	nd	0.178	0.593		
<b>Full score Biagi's compliance test for GFD; %</b>	NA	84.2	91.9	nd		.326		
<b>Daily smoking; %</b>	5.8	10.5	6.5	nd	0.605	0.621		
<b>Other autoimmune disease; %</b>	10.0	21.1	35.5	nd	0.247	0.276		
<b>GSRS-IBS; median (25% , 75%), range 13 - 91</b>	22.0 (13.75 , 29.50)	26.0 (19.0 , 36.0)	20.5 (17.3 , 31.5)	nd	0.087	0.217		
<b>CRP; median, median (25% , 75%), mg/L</b>	1.3 (<0.6 , 4.8)	1.3 (<0.6 , 4.2)	1.0 (<0.6 , 2.0)	0.9 (<0.6 , 3.8)	0.592	0.689	0.419	0.308
<b>Anti-TG2 IgA; median (25% , 75%), units/mL</b>	<1.0 (<1.0 , <1.0)	<1.0 (<1.0 , <1.0)	<1.0 (<1.0 , <1.0)	9.0 (4.9 , 24.7)	0.217	<b>0.024</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<b>Anti-DGP IgG; median (25% , 75%), units</b>	<5.0 (<5.0 , <5.0)	<5.0 (<5.0 , <5.0)	<5.0 (<5.0 , 8.3)	41.5 (20.0 , 93.3)	0.114	<b>0.002</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<b>Frequency of gluten-specific cells; median (25% , 75%)</b>	0.19 (0.05 , 0.44)	0.10 (0.07 , 0.20)	3.09 (1.03 , 7.01)	5.46 (2.63 , 8.90)	0.050	<b>&lt;0.001</b>	0.058	<b>&lt;0.001</b>
<b>Variable 1; median (25% , 75%)</b>	0.95 (0.64 , 1.31)	0.78 (0.54 , 0.99)	2.35 (1.61 , 3.54)	3.09 (2.07 , 4.79)	0.092	<b>&lt;0.001</b>	<b>0.046</b>	<b>&lt;0.001</b>
<b>Variable 2; median (25% , 75%)</b>	1.23 (0.85 , 2.29)	0.91 (0.58 , 1.14)	2.94 (2.54 , 3.58)	2.71 (2.11 , 3.61)	0.079	<b>&lt;0.001</b>	0.378	<b>0.001</b>
<b>Variable 3; median (25% , 75%)</b>	1.11 (0.88 , 1.65)	0.78 (0.74 , 1.03)	1.59 (1.30 , 1.94)	1.50 (1.29 , 1.87)	<b>0.001</b>	<b>&lt;0.001</b>	0.864	0.071
<b>CD38<sub>RR</sub>; median (25% , 75%)</b>	1.82 (<0.01 , 2.99)	NA	0.43 (0.08 , 1.89)	5.80 (3.30 , 10.35)			<b>&lt;0.001</b>	<b>&lt;0.001</b>

NA, not applicable; nd, not done; BMI, body mass index. CRP, C-reactive protein. See main text for further explanation of the variables.

Significant P-values (< .05) are in bold numbers. Fisher's exact test was used in the case of ratios. Variables that were normal distributed before or after transformation were tested with two sample T-tests, and non-parametric variables were tested with Mann-Whitney test.

## Supplementary tables and supplementary figure legends:

Supplementary Table 2. Follow-up evaluation of 6 control subjects

ID	Age	Gender	Biopsy result Marsh type	Anti-TG2 IgA <sup>#</sup>	Anti-DGP IgG <sup>#</sup>	Diagnosis of celiac disease	Initial Anti-TG2 IgA <sup>#</sup>	Initial Anti-DGP IgG <sup>#</sup>	Initial HLA-DQ–gluten test
39	33	M	1 – 3A*	2.9	28	Not given	2.8	25	+
59	33	M	0	<1	<5	Not given	<1	<5	-
87	30	F	2 – 3a / 3a**	5.5	23	Given	12.4	31	+
89	54	F	1	2.1	12	Not given	10.2	22	+
107	23	F	0	<1	<5	Not given	<1	6	-
149	28	F	0 / 3b**	1	24	Given	1.3	29	+

Duodenal histology and serology was done 6 – 12 months after the index test in 4 index-test positive controls and 2 index-test negative controls, all with a frequency of gluten-specific T-cells equivalent to the level in UCD-subjects. Results from this follow-up evaluation are shown here together with the initial index test results (last 3 columns).

<sup>#</sup> Reference range; anti-TG2 IgA < 3 units, anti-DGP-IgG < 20 units/mL

\* The duodenal biopsy showed focal increase in IEL and focal villous changes.

\*\* The value before the slash is the Marsh score in the second part of duodenum (descending duodenum) and the value after the slash is the Marsh score in the first part of duodenum (the duodenal bulb).

Supplementary Table 3. Analytical and biological variation

Model	Status (n)	Analytical difference (Mean)	Analytical variation (SD)	Biological variation (SD)
GS – TCD	TCD (5)	-0.005	0.045	0.061
Control – UCD	Controls (5)	0.002	0.099	0.086

The reproducibility (analytical variation) and biological variation for the HLA-DQ–gluten tetramer test was examined in 10 subjects, five TCD-subjects and five controls. Blood was taken twice with one week interval to calculate the standard deviation (SD) for weekly biological variation and at each time point the sample was divided into two halves to determine the mean analytical difference and analytical SD.

Supplementary Figure 1. **Overview of recruitment, inclusion and index test results.**

Supplementary Figure 2. **Characterization of healthy control participants with increased numbers of HLA-DQ:tetramer-binding cells.** Four participants in the control group with frequency of gluten-specific T-cells equivalent to the UCD-group and who had celiac disease excluded by a follow-up duodenal histology (see supplementary table 2), were re-tested 6 – 12 months later and could display a persisting high frequency of gluten-specific T cells. (B) The gluten-specific T cells were sorted and cultured as T-cell lines without antigenic stimulation in vitro. The reactivity of the T-cell lines to the antigens native gluten, deamidated gluten and five different gluten peptides separately with epitopes represented in the five HLA-DQ–gluten tetramer molecules were tested in T-cell proliferative assays by assessing 3H-thymidine incorporation. The proliferative responses are given as stimulation indices (ratios of responses with antigen over no antigen), and a stimulation index over 3 is considered positive (horizontal dotted line).

Supplementary Figure 3. **CD38-expression on gluten-specific T-cells as a marker for gluten intake in celiac disease patients.** (A) Frequency of CD38<sup>+</sup> gluten-specific T-cells in all groups. (B) Ratio of CD38<sup>+</sup> cells in HLA-DQ–gluten-tetramer<sup>+</sup>β7<sup>+</sup>T<sub>EM</sub> divided by the same ratio in the HLA-DQ–gluten tetramer<sup>+</sup>β7<sup>+</sup>T<sub>EM</sub> (CD38<sub>RR</sub>) in TCD and UCD groups. (C) Receiver operating characteristics (ROC) curve for CD38<sub>RR</sub> in TCD and UCD groups. The dotted lines show the cut-off for optimal sensitivity and specificity, also shown as the horizontal dotted line in panel (B). Open circles represent participants with an elevated antibody titer (anti-TG2 IgA > 3 units/ml or anti-DGP IgG > 20 units). AUC; area under the ROC-curve.

Supplementary Figure 4. **Analytical and weekly variation of the HLA-DQ–gluten tetramer test.** (A and B) The average of two split samples taken at the same time point is shown for

both models and illustrated the analytical variation. (C and D) The average of the two split samples is plotted with one week interval to illustrate the weekly variation.

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