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Synthetic Neoepitopes of the Transglutaminase–Deamidated Gliadin Complex as Biomarkers for Diagnosing and Monitoring Celiac Disease

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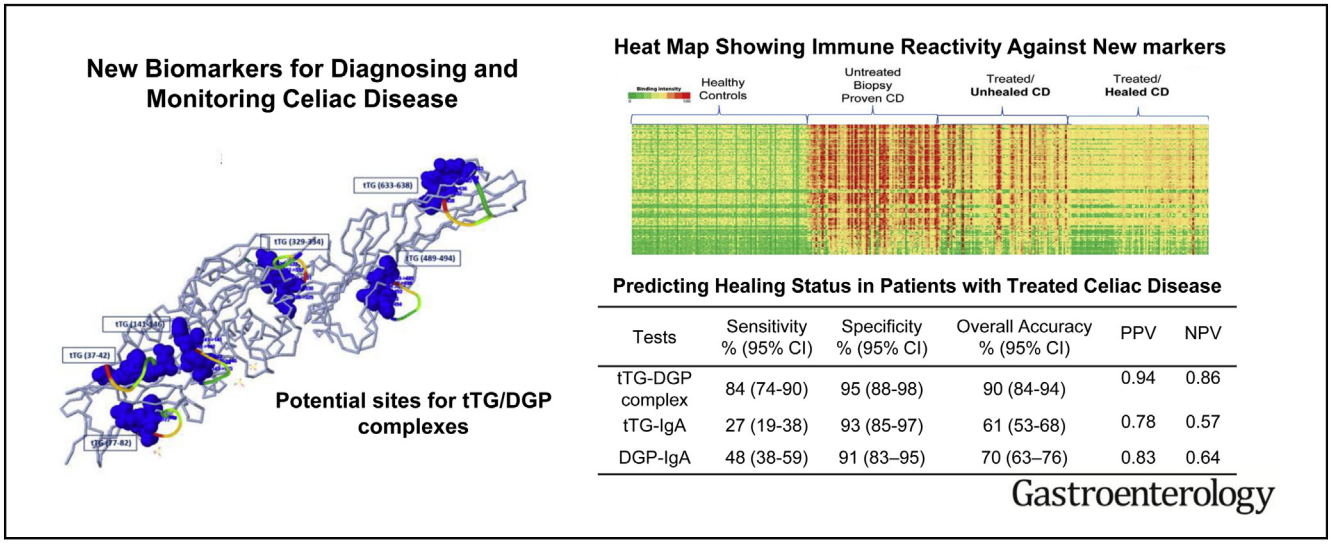

See Covering the Cover synopsis on page 526.

BACKGROUND & AIMS: Celiac disease (CeD) has characteristics of an autoimmune disease, such as increased antibody levels to tissue transglutaminase (tTG). However, assays to measure these biomarkers in blood samples do not identify patients with sufficient accuracy for diagnosis or monitoring of CeD. We aimed to discover biomarkers of CeD derived from neoepitopes of deamidated gliadin peptides (DGP) and tTG fragments and to determine if immune reactivity against these epitopes can identify patients with CeD with mucosal healing. **METHODS:** We analyzed serum samples from 90 patients with biopsy-proven CeD and 79 healthy individuals (controls) for immune reactivity against the tTG-DGP complex (discovery cohort). A fluorescent peptide microarray platform was used to estimate the antibody-binding intensity of each synthesized tTG-DGP epitope. We validated our findings in 82 patients with newly diagnosed CeD and 217 controls. We tested the ability of our peptide panel to identify patients with mucosal healing (based on the histologic analysis) using serum samples from patients with treated and healed CeD (n = 85), patients with treated but unhealed CeD (n = 81; villous atrophy despite a adhering a gluten-free diet), patients with untreated CeD (n = 82) and disease controls (n = 27), villous atrophy without CeD), and healthy controls (n = 217). Data were analyzed using principal component analysis followed by machine learning and support vector machine modeling. **RESULTS:** We identified 172 immunogenic epitopes of the tTG-DGP complex. We found significantly increased immune reactivity against these epitopes vs controls. In the both cohort, the set of neoepitopes derived from the tTG-DGP complex identified patients with CeD with 99% sensitivity and 100% specificity. Serum samples from patients with untreated CeD had the greatest mean antibody-binding intensity against the tTG-DGP complex (32.5 ± 16.4). The average antibody-binding intensity was significantly higher in serum from patients with treated but unhealed CeD mucosa (15.1 ± 7.5) than in patients with treated and healed CeD mucosa (5.5 ± 3.4) ($P < .001$). The assay identified patients with mucosa healing status with 84% sensitivity and 95% specificity. **CONCLUSIONS:** We identified immunogenic epitopes of the tTG-DGP complex, and found that an assay to measure the immune response to epitopes accurately identified patients

with CeD, as well as patients with mucosal healing. This biomarker assay might be used in detection and monitoring of patients with CeD.

Keywords: Noninvasive Marker; Diagnostic; Follow-up; Response to Treatment.

Celiac disease (CeD) has the features of an autoimmune disease, such as increased antibody levels to the self-antigen tissue transglutaminase (tTG) that return to normal when adhering to a gluten-free diet (GFD).[1] The adaptive response of CeD consists of T-cell– and B-cell–mediated responses to gliadins and similar proteins in wheat, barley, and rye.[2,3] Currently, the primary serologic markers of CeD are antibodies to tTG and gliadin peptides (GPs) that have been deamidated by tTG.[4,5] Autoimmunity (characterized by anti-tTG antibodies) is uniquely dependent on the continued ingestion of gluten.[6–8] Several studies showed that deamidated gliadin-derived peptides (DGPs) that have been modified by tTG are more immunogenic in patients with CeD than native GPs, which have not been modified by tTG.[9,10] Furthermore, Sollid et al.[11] proposed the hapten-carrier theory to explain autoimmunity initiation in patients with CeD. Compatible with this theory, a complex consisting of small parts of DGPs and tTG may elicit or augment an immune response in CeD. Furthermore, neoepitopes from the tTG-DGP complex were suggested as accurate diagnostic markers of CeD.[12,13] However, this association between tTG and DGPs has not been fully established.

Serologic tests have shown high sensitivity and specificity for diagnosing untreated CeD, especially tests for tTG–immunoglobulin A (tTG-IgA), but less so for DGP-IgA and DGP–immunoglobulin G, but upper endoscopy with biopsy of the duodenum is required to confirm diagnosis because of variability in CeD serology.[1] Moreover, a reference tTG-IgA level after starting a GFD is a poor predictor of intestinal healing.[14] The only accurate method for verifying intestinal healing is to histologically evaluate a biopsy of the duodenum, which is invasive and expensive. Previously, we developed an ultrahigh-density protein and peptide array that enables comprehensive interrogation of the antibody responses to native peptides, DGPs, and tTG.[3] Thus, in this study we aimed to explore epitope recognition in serum samples from patients with untreated CeD, in particular immune recognition of novel combinations of tTG and DGPs, and to further determine whether the antibody recognition patterns of these peptides are predictive of mucosal healing in patients with treated CeD.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Serological tests for celiac disease (CeD), including anti tissue transglutaminase-IgA (tTGA) and anti deamidated gliadin peptides-IgA (DGP), are currently used for diagnosing and monitoring of CeD; however, the accuracy of these biomarkers is insufficient.

NEW FINDINGS

The authors identified immunogenic epitopes of the tTG-DGP complex, and found that an assay to measure the immune response to epitopes accurately identified patients with celiac disease, as well as patients with mucosal healing.

LIMITATIONS

The deamidation of gliadin was not generated by direct incubation with tTG. Unhealed mucosa may be related to ongoing exposure to a gluten-containing diet.

IMPACT

Identified neoepitopes derived from the tTG-DGP complex could be clinically utilized, since persistent immune responses to these epitopes is a promising noninvasive predictor of persistent mucosal injury in patients with treated CeD.

CLINICAL AT

Methods

This analysis is composed of 2 case-control studies. The first study aimed to identify a potential biomarker derived from novel combinations of tTG and DGPs for diagnosing CeD. The second study further evaluated the biomarker identified in the first study to differentiate healing status in patients with CeD who were adhering to a GFD. Our study was approved by the Institutional Review Board of Mayo Clinic in Rochester, Minnesota.

Study Population

Cohorts for identifying diagnostic markers (Supplementary Tables 1 and 2). To discover potential biomarkers for CeD, 90 patients with biopsy-proven CeD and 79 healthy control patients comprised the exploratory population, from which serum samples were collected in our previous study.[3] Another cohort of 82 patients with newly diagnosed CeD and 217 control patients whose serum samples were prospectively collected was used as the validation cohort to verify the diagnostic utility of the biomarker discovered in the first exploratory cohort. Among 82 patients with newly diagnosed CeD in the validation set, 4 patients with IgA deficiency were included.

Cohorts for disease monitoring. To evaluate the identified biomarker for predicting mucosal healing status in patients with treated CeD, serum samples were prospectively collected from patients with treated and healed CeD mucosa (n = 85), patients with treated but unhealed CeD mucosa (n = 81), patients with untreated CeD mucosa (n = 82), and control patients (n = 217). Mucosal healing status was defined by persistent villous atrophy despite adhering to a GFD or histologic recovery (no villous atrophy). Patients with refractory

Abbreviations used in this paper: **CeD, celiac disease; DGP, deamidated gliadin-derived peptide; Fmoc, fluorenylmethoxycarbonyl; GFD, gluten-free diet; GP, gliadin peptide; IgA, immunoglobulin A; ROC, receiver operating characteristic; tTG, tissue transglutaminase.**

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CeD were not included in this study. The mucosal healing status in the small intestine was classified based on the pathologic reports; treated patients with CeD who had partial or total villous atrophy were categorized into treated but unhealed CeD group.

Controls with villous atrophy but no CeD (disease controls). To compare the immune reactivity against epitopes of DGP, tTG, and tTG-DGP complex, we also tested serum samples of selected disease controls who were diagnosed with autoimmune enteropathy (n = 10), common variable immune deficiency associated enteropathy (n = 6), or drug-induced spruelike enteropathy (n = 11).

Peptide Synthesis

The peptide array was described in our previous study.[3] Briefly, for solid-phase peptide synthesis, silicon-based wafers (300-mm diameter), with a 100-nm-tall, thermal oxide–coated feature area and nonfeature area containing silicon, were made using photolithography and an inductively coupled plasma deep-etching technique. The surface of the prepared silicon-based wafer contained a monolayer of aminosilane that provided peptide attachment sites, in which peptide synthesis was performed using standard fluorenylmethoxycarbonyl (Fmoc) chemistry. After Fmoc protection was removed, the unprotected amine was coupled with the incoming desired Fmoc amino acid using a specific reticle that activates only the desired site where the incoming amino acid needs to be coupled. The process was repeated for each individual layer of amino acids to create the desired peptide sequences at each feature area.

tTG and the tTG-DGP Complex

In the previous study,[3] 12-mer peptides, with sequences from a lateral shift of 2 amino acids in α, β, γ, and Ω fractions of gliadin, were synthesized on silicon-based wafers. In addition, in these synthetic GPs, each glutamic acid was replaced in the position of glutamine, mimicking the deamidation of GPs (DGPs). The peptide microarray immunoassay was used to assess native peptides, DGPs, and key 3-mer GP sequences with high antibody-binding intensity associated with CeD.[3] Similar to GPs, overlapping 12-mer peptides and various lengths of tTG were synthesized. For the main purpose of this study, novel combined sequences, which were combinations of key 3-mer GP sequences and tTG subsequences, were synthesized on the silicon-based wafers. For example, in the new combined sequence YGDGVS**QPEQPF**, YGDGVS is from tTG (positions 245–250) and **QPE** and **QPF** are key 3-mer GP sequences. The basic method for selecting the new combined tTG-DGP sequences is shown in Figure 1.

Statistical Analysis

A fluorescent peptide microarray platform (Vibrant Sciences, San Carlos, CA) was used to estimate the antibody-binding intensity of each synthesized tTG-DGP neoepitope. The region of interest stitching program using JAVA transformed an image file from the scan of a peptide microarray chip to individual antibody-binding intensity values, which were calculated using the median foreground intensity and then applying binary log transformation to stabilize variance. Each antibody-binding intensity value is linked to a corresponding peptide sequence. A random forest was used to remove the unreliable peptide sequences of the tTG-DGP complex.[15] A random forest classifier was trained to detect areas of peptide sequences with values that were not within the 95% linear regression confidence band of a single linear regression analysis of multiple assays (performed using the rapmad [Robust Analysis of Peptide MicroArray Data] R-package).[16] Furthermore, background normalization modeling was also applied, which was performed using an expectation-maximization algorithm (performed using R-package) that placed blank spots where no sequences were synthesized. After eliminating background noise and unreliable peptide sequences, support vector machine modeling[17] was applied to the training set to construct a hyperplane and maximize the margins of the training data between the 2 classes (CeD vs no CeD) (performed using the Python package), with the aim of identifying the disease-associated peptide sequences of the tTG-DGP complex. Based on results of the support vector machine training, the identified disease-associated peptide sequences were then tested on unknown samples to compute the prediction accuracy, sensitivity, and specificity. Further receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity of each peptide. The threshold value for the ROC curve of each peptide was determined by choosing the value with the highest sensitivity and specificity. Furthermore, principal component analysis, hierarchical cluster analysis with heat maps, and random forest

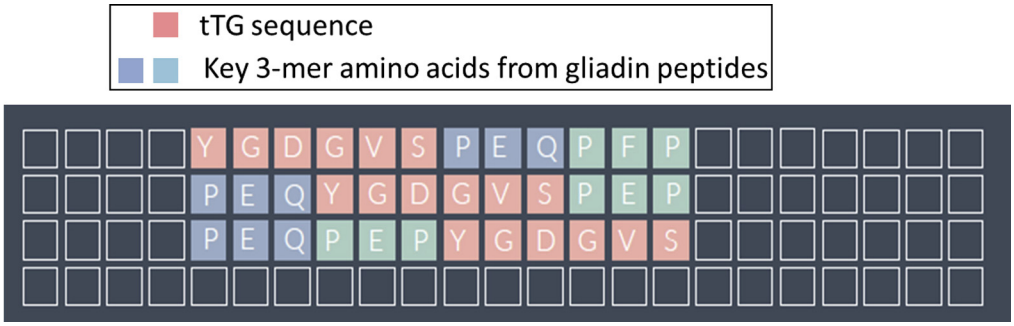

Figure 1. Combined epitopes of the tTG-DGP complex. Examples of 3 different ways to combine tTG and GP segments. YGDGVS is located at positions 245 to 250 of the tTG peptide, and PEQ and PEP are 2 key 3-mer amino acids of gliadin. *Upper row*, YGDGVS is followed by PEQ and PEP. *Middle row*, YGDGVS is located between PEQ and PEP. *Lower row*, PEQ and PEQP are followed by YGDGVS. E indicates glutamic acid; Q, glutamine; Y, Tyrosine; D, Aspartate; G, Glycine; V, Valine; P, Proline; F, Phenylalanine; S, Serine.

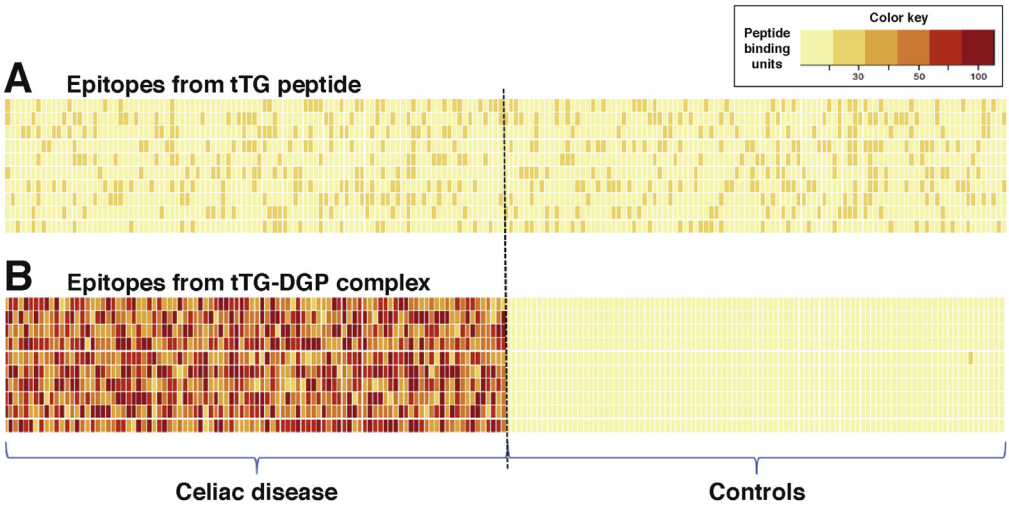

Figure 2. Heat maps showing immune reactivity against tTG and the tTG-DGP. (*A*) Immune reactivity against the tTG peptide. No significant differences in immune reactivity were found between the serum samples from patients with CeD and control patients. (*B*) Immune reactivity against the neoepitopes of the tTG-DGP complex. The antibody-binding intensity of the neoepitopes of the tTG-DGP complex was significantly increased in the serum samples of the patients with celiac disease, but immune reactivity was minimal or nearly 0 in controls.

multivariate analysis were performed using the R or Python package.[18]

Results

Diagnostic Accuracy of the tTG-DGP Complex

The synthesized tTG peptide fragments were tested in serum samples obtained from 90 patients with CeD and 79 control patients to determine immune reactivity against tTG fragments. Interestingly, immune reactivity against the tTG fragments was not significantly increased in patients with CeD compared with control patients (Figure 2*A*). Because GPs can form complexes with tTG in the duodenal mucosa of patients with CeD,[19] it is plausible that an adaptive immune response against the tTG-DGP complex would be generated. Thus, we synthesized 12-mer neoepitopes derived from tTG and key 3-mer motifs of native peptides or DGPs. These neoepitopes were tested in the serum samples of patients with CeD and control patients to identify immunogenic epitopes, which were defined as any sequence with an area under the ROC curve value >0.7. Finally, a total of 172 immunogenic epitopes of the tTG-DGP complex were identified (Supplementary Table 3). Figure 2*B* shows significantly increased immune reactivity against the neoepitopes of the tTG-DGP complex in patients with CeD compared with control patients. In the training cohort, the identified set of neoepitopes derived from the tTG-DGP complex showed very high sensitivity (99%) and specificity (100%) for diagnosing CeD. To validate the discriminative power of this tTG-DGP complex set, serum samples from a validation cohort of 82 patients with CeD and 217 control patients were assayed in a blind test. Encouragingly, this tTG-DGP complex set showed high accuracy for distinguishing CeD cases from controls, achieving 99% sensitivity and 100% specificity. In particular, compared with current serologic tests for CeD, including tTG-IgA and DGP-IgA, sensitivity and specificity were higher when using these neoepitopes to differentiate CeD cases from controls (Table 1). Serum samples from patients diagnosed with selected control diseases in which enteropathy is present in the absence of CeD, also were tested. The control disease patients consisted of 10 patients with autoimmune enteropathy, 6 patients with common variable immunodeficiency–associated enteropathy, and 11 patients with drug-induced enteropathy. We found that the immune reactivity against neoepitopes of tTG-DGP complex in these disease controls was significantly lower than in patients with CeD and was similar to other control patients. Of interest was that 4 patients with CeD with complete IgA deficiency had no immune reactivity against neoepitopes of tTG-DGP complex.

Table 1. Sensitivity, Specificity, and Overall Accuracy of Using the tTG-DGP Complex to Diagnose CeD

Peptide/Protein	Sensitivity % (95% CI)	Specificity % (95% CI)	Overall Accuracy % (95% CI)	PPV	NPV
tTG-DGP complex	99 (93–100)	100 (98–100)	99 (98–100)	1	0.99
tTG-IgA[a]	90 (82–95)	99 (96–100)	97 (94–98)	0.97	0.96
DGP IgA (ELISA)[a]	91 (83–96)	97 (94–98)	97 (94–98)	0.96	0.97

ELISA, enzyme-linked immunosorbent assay; NPV, negative predictive value; PPV, positive predictive value.
[a]Determined using the tTG-IgA or DGP-IgA ELISA test (Inova Diagnostics, San Diego, CA).

Table 2. Characteristics of the Patients With Treated CeD According to Mucosa Healing Status

Characteristic	Treated/healed CeD mucosa (n = 85)	Treated/unhealed CeD mucosa (n = 81)	*P*
Age at diagnosis, mean (SD), y	41.1 (15.2)	47.5 (15.5)	<.001
Female sex, %	73	72	.60
Duration of gluten-free diet, median (IQR), *y*	2.8 (1.7–5.1)	3.5 (1.8–8.1)	.16[a]
tTG-IgA positivity, %	7	27	<.001
DGP-IgA positivity, %	9	48	<.001
Partial or total villous atrophy, %	0	100	<.001

SD, standard deviation.
[a]Determined using nonparametric tests.

tTG-DGP Complex and Disease Activity in Patients With Treated CeD Mucosa

Table 2 shows the characteristics of treated patients with CeD according to mucosal healing status. Patients with treated and healed CeD mucosa were younger on average than patients with treated but unhealed CeD mucosa, but similar with regard to sex (73% vs 72% of patients were women, respectively). Interestingly, patients with treated but unhealed CeD mucosa adhered to a GFD longer than patients with treated and healed CeD, but this was not statistically significant ($P = .16$). Although 7% of patients with treated and healed CeD mucosa were positive for tTG-IgA, 27% of patients with treated but unhealed CeD mucosa were positive for tTG-IgA, and approximately three-quarters of patients with treated but unhealed CeD mucosa were negative. In addition, 48% of patients with treated but unhealed CeD mucosa were positive for DGP-IgA and 9% of patients with treated but healed CeD mucosa were positive for DGP-IgA.

Figure 3 shows immune reactivity against the neoepitopes of the tTG-DGP complex in patients with treated CeD according to healing status. Overall, as shown in the heat map, immune reactivity against the neoepitopes of the DGP-tTG complex was stronger in patients with treated but unhealed CeD mucosa than patients with treated and healed CeD mucosa and control patients (Figure 3*A*). Interestingly, the average antibody-binding intensity of the neoepitopes derived from the tTG-DGP complex significantly differed among the 5 groups ($P < .001$). Immune reactivity decreased stepwise according to intestinal mucosal damage status, showing the highest mean (standard deviation) reactivity in the patients with untreated CeD mucosa (32.5 [16.4]) followed by patients with treated but unhealed CeD mucosa (15.1 [7.5]), patients with treated and healed CeD mucosa (5.5 [3.4]), control patients (1.3 [0.5]), and disease controls (1.3 [0.4]). Furthermore, in the principal component analysis (Figure 3*B*), the patients with treated and healed CeD mucosa and control patients were closely aggregated, but the patients with treated and unhealed CeD mucosa and patients with untreated CeD mucosa were similarly distributed.

Figure 4 shows the potential utility of the neoepitopes of the tTG-DGP complex to diagnose treated but unhealed CeD mucosa compared with the tTG-IgA enzyme-linked immunosorbent assay. Although approximately 75% of patients with treated but unhealed CeD tested negative for tTG-IgA, most of these patients showed increased immune reactivity against the neoepitopes of the tTG-DGP complex. Compared with the tTG-IgA enzyme-linked immunosorbent assay, the neoepitopes of the tTG-DGP complex showed higher sensitivity (84%) and specificity (95%) with a positive predictive value of 0.94 and a negative predictive value of 0.86 for predicting healing status in patients with treated CeD mucosa (Table 3).

Discussion

Serologic tests for CeD, especially tTG-IgA tests, have high sensitivity and specificity, but biopsy of the small intestines is still considered the definitive method for diagnosing CeD. In addition, no useful noninvasive markers exist for monitoring disease activity in patients with CeD who have started a GFD. In this study, we found potential biomarkers for CeD, synthesized neoepitopes derived from DGP and tTG fragments, that show better diagnostic accuracy than current serologic tests for distinguishing patients with CeD from controls. Intriguingly, these neoepitopes showed more significant reactivity in the serum samples of the patients with treated but unhealed CeD mucosa compared with the patients with treated and healed CeD mucosa or control patients. In addition, immune reactivity against these neoepitopes was somewhat less in patients with treated but unhealed CeD mucosa than patients with untreated CeD. This distinctive increase in immune reactivity was still prominent in patients with CeD testing negative for tTG-IgA who have treated but unhealed mucosa.

Serologic tests for CeD have been extensively investigated and are considered an effective first step in diagnosing CeD.[5,20–25] Recent European guidelines suggested that sufficiently and strongly positive serologic tests for CeD, including tests for tTG-IgA and endomysial antibody, are enough to confirm CeD; therefore, biopsy of the small intestines may not be needed to diagnose CeD in this subgroup.[26] However, the results of serologic tests vary greatly across different settings and populations,[5,22,24,25] and most guidelines still recommend intestinal biopsy to reach the final diagnosis of CeD.[1,27,28] Especially the positive predictive values of CeD serologic tests are relatively low because of the low prevalence of CeD. In addition, for patients with

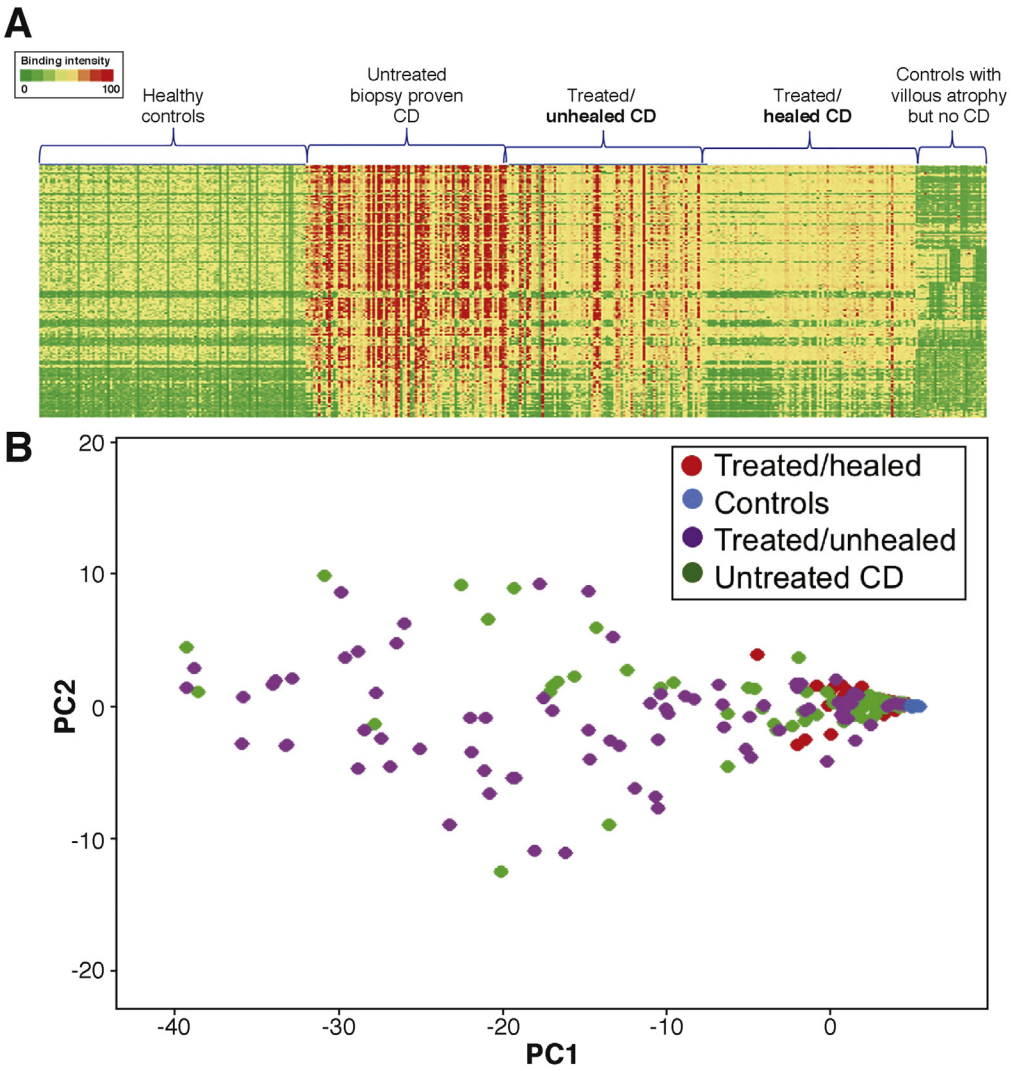

Figure 3. Immune reactivity against epitopes of the tTG-DGP complex based on antibody-binding intensity. (*A*) Immune reactivity against epitopes of the tTG-DGP complex in patients with CeD and control patients shows higher antibody-binding intensity in patients with untreated CeD and patients with treated and unhealed CeD; but low antibody-binding intensity in patients with treated and healed CeD, healthy controls, and disease controls who had villous atrophy due to autoimmune enteropathy, common variable immunodeficiency–associated enteropathy, or drug-induced enteropathy. (*B*) Principal component analysis of immune reactivity against neoepitopes of the tTG-DGP complex. Biplot illustrates the correlation between the level of immune reactivity against the tTG-DGP complex and CeD phenotype. Treated/healed CeD group (*red dots*) and healthy controls (*blue dots*) appear together on the principal component analysis plot. In the figure, CD indicates celiac disease.

selective IgA deficiency that is more commonly associated with CeD than in the general population, the tTG-IgA test was not effective to diagnose CeD. All 4 patients with selective IgA deficiency were negative for tTG-IgA but showed increased immune reactivity against the neoepitopes of the tTG-DGP complex. Furthermore, patients who had intestinal villous atrophy but no CeD showed no immune reactivity against the neoepitopes of tTG-DGP complex. In the present study, the neoepitopes of the tTG-DGP complex showed comparable or even higher diagnostic accuracy for discriminating CeD than clinically available serologic tests. Indeed, several studies show that gliadin directly binds to tTG in the duodenal mucosa of patients with CeD, and the cross-linking of GPs by tTG has been suggested to be involved in the development of CeD.[12,19,29] Interestingly, we found that immune reactivity against linear epitopes of tTG was not increased in patients with CeD, suggesting that the linear epitopes of tTG may not be recognized in the sera of CeD patients. The formation of the tTG-GP complex could be an important step in the development of autoimmunity in persons with CeD, indicating epitope spread from gliadin to tTG, but the reason for the development of autoimmunity against tTG in patients with CeD is unknown.[30] In addition, few studies have tested using cross-linked tTG and GPs as biomarkers for CeD, even though diagnostic accuracy was low in these studies compared with our study.[30–32]

Although a GFD is an effective therapy for CeD, patients with CeD frequently find it difficult to adhere to a GFD,

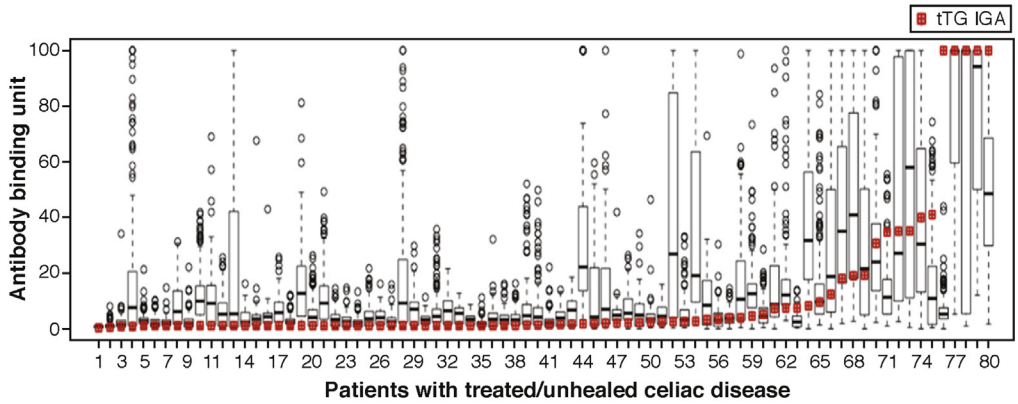

Figure 4. Antibody-binding levels of tTG-IgA vs tissue transglutaminase–derived gliadin peptide complex in patients with treated but unhealed CeD. For most patients, the levels of tTG-IgA are low but the neoepitopes to tTG-DGP complex exhibit higher antibody-binding levels. Patients with high tTG-IgA, which was depicted by *red check box*, showed higher antibody-binding levels of neoepitopes of tTG-DGP complex. The box plot indicates the antibody-binding levels to neoepitopes of tTG-DGP complex; the horizontal line of the box indicates patients with treated but unhealed CeD mucosa. The *red check box* depicts the titers of tTG-IgA.

resulting in ongoing intestinal damage. Several studies have shown that persistent mucosal damage in patients with treated CeD mucosa was associated with several severe complications, including lymphoproliferative malignancy, bone diseases,[33,34] and possibly excess mortality.[35,36] Similar to other chronic conditions, disease monitoring in patients with treated CeD mucosa is necessary. Follow-up biopsy of the duodenum is considered the gold standard of care for treated CeD, although it is both invasive and expensive. Although several serologic tests for CeD are also recommended for monitoring CeD,[27,37–39] the results of these serologic tests are not well correlated with intestinal mucosal healing status in patients with treated CeD.[40,41] A recent meta-analysis reported that serologic tests for CeD, including tests for tTG-IgA and endomysial antibody, have low sensitivity (less than 50%) compared with follow-up biopsy for detecting persistent villous atrophy in patients with CeD who adhere to a GFD, indicating the need for more accurate noninvasive markers for monitoring CeD. Compared with tTG-IgA, DGP-IgA has been shown to be a better predictor of healing status in patients with treated CeD; however, the sensitivity and specificity of DGP-IgA were not optimal in our study. We tested the identified neoepitopes of the tTG-DGP complex to determine whether these neoepitopes predict persistent mucosal damage in patients with treated CeD and found much higher sensitivity and specificity for predicting healing status in patients with treated CeD compared with current serologic tests in our study. In particular, immune reactivity against the neoepitopes of the tTG-DGP complex was still high in patients with treated but unhealed CeD whose tTG-IgA titers were normalized. Thus, these neoepitopes could be good biomarkers for determining healing status in patients with treated CeD mucosa. Immune reactivity against the neoepitopes of the tTG-DGP complex cannot take the place of the necessity of intestinal biopsies when monitoring disease activity because a small portion of patients with CeD can progress to lymphoproliferative disorders or type II refractory CeD, which are associated with aberrant T lymphocytes; however, increased immune reactivity may give clues for ongoing inflammation with persistent intestinal damage. Thus, with such a good positive predictive value (94%) to predict unhealed mucosa, this new test would provide the possibility of avoiding intestinal biopsies if the treated patient still shows the increased immune reactivity against neoepitopes of tTG-DGP complex. Furthermore, because the negative predictive value of the new test (86%) was high, if negative, a need for biopsy in patients with treated CeD may be obviated.

The mechanism that results in persistent immune reactivity against the neoepitopes of the tTG-DGP complex in patients with CeD who are adhering to a GFD and have

Table 3. Sensitivity, Specificity, and Overall Accuracy of Using tTG-DGP Complex, tTG-IgA, and DGP-IgA to Predict Healing Status in Patients With Treated CeD

Peptide/Protein	Sensitivity % (95% CI)	Specificity % (95% CI)	Overall Accuracy % (95% CI)	PPV	NPV
tTG-DGP complex	84 (74–90)	95 (88–98)	90 (84–94)	0.94	0.86
tTG-IgA[a]	27 (19–38)	93 (85–97)	61 (53–68)	0.78	0.57
DGP-IgA[a]	48 (38–59)	91 (83–95)	70 (63–76)	0.83	0.64

NPV, negative predictive value; PPV, positive predictive value.
[a]Determined using the tTG-IgA or DGP-IgA enzyme-linked immunosorbent assay (Inova Diagnostics, San Diego, CA).

mucosal atrophy is unclear, especially in patients whose tTG-IgA titers were already normalized. Because persistent intestinal villous atrophy is more commonly associated with poor adherence to a GFD in patients with treated CeD mucosa,[35,42] even a small amount of gluten can maintain the immune response in a person adhering to a GFD. In our study, some treated patients were still positive for tTG-IgA, and, interestingly, these patients showed much higher immune reactivity against neoepitopes of tTG-DGP complex than patients with negative tTG-IgA. Several studies have also demonstrated the persistence of DGP-IgA in patients with CeD who adhered to strict GFD for at least 1 year.[43–46] Furthermore, Spatola et al[45] recently showed that the persistence of antibodies against DGP was associated with nonresponsive CeD in patients with treated CeD, even though the sample size of nonresponsive CeD cases was small. As with other autoimmune diseases,[47–51] epitope spreading may occur in patients with CeD, especially from GPs to tTG. As exposure to the evoking antigens declines in patients with CeD, immunity against self- and non–self-antigens also disappears in a reverse manner. Thus, it is conceivable that immune reactivity against the tTG-DGP complex is highly correlated with mucosal healing in patients with treated CeD mucosa.

Our study has limitations. First, GPs were not deamidated by tTG in a biologic process; rather, these peptides were synthesized using all possible substitutions of specific glutamine residues. Second, we did not determine any experimental 3-dimensional structures of the tTG-DGP complex. However, in this study, the synthesized neoepitopes of the tTG-DGP complex were short (confined to approximately 12 amino acid residues) and mimicked antibody recognition of DGPs and tTG (ie, the antibodies needed to detect only a single patch on the key binding residues).[52,53] Furthermore, the synthesized peptides on the microarray adapted to the 3-dimensional conformational requirements for reactions between antibodies and epitopes.[53] Thus, it is conceivable that the synthesized neoepitopes of the tTG-DGP complex had the specific key binding residues that evoked the immune responses by the antibodies formed in patients with CeD. In addition, patients with treated but unhealed mucosa can be related to gluten exposure so that immune response to exposed gluten may be still persisted in these patients.

In conclusion, the neoepitopes derived from the tTG-DGP complex are extremely accurate predictors of untreated CeD mucosa, and persistent immune response to these epitopes is a promising noninvasive predictor of persistent mucosal injury in patients with treated CeD. These data also suggest that, unlike antibodies to tTG, these antibodies persist long after treatment in patients with CeD with nonhealing mucosa.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.10.025.

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Conflicts of interest
Joseph A. Murray has received grant support from the National Institutes of Health, ImmunogenX, and Allakos. He serves on the advisory board of Celimmune, LLC; was a consultant to Intrexon, Bioniz, Lilly, Amgen, Innovate. Sonomaceuticals, LLC, GlaxoSmithKline, Genentech, Inc., and Glenmark Pharmaceuticals Ltd.; serves as a consultant to Sanofi; and has equity options in Torax Medical, and roylaties from Evolo. The remaining authors have no conflicts to disclose. J. J. Rajasekaran, Vasanth Jayaraman, Kang Bei, Hari Krishnamurthy, Tianhao Wang, Karenah E. Rajasekaran, Karthik Krishna, and Hari Krishnan Krishnamurthy are employed by Vibrant Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Institutes of Health.

Supplementary Table 1. Clinical Characteristics of the Exploratory Population

		Age, *y*		Sex, %	
Group	N	Mean	Range	Male	Female
Celiac disease	90	39.4	19.5–60.2	43	57
Healthy controls	79	40.2	19.7–63.3	48	52

Supplementary Table 2. Clinical Characteristics of the Validation Population

		Age, *y*		Sex, %	
Group	n	Mean	Range	Male	Female
Celiac disease	82	47.7	34.0–59.0	28.0	72.0
Healthy controls	217	35.6	26.6–44.6	40.1	59.9

Supplementary Table 3. List of tTG–DGP Complex Sequences Showing High Sensitivity and Specificity for Identifying CeD in Healthy Control Patients With Area Under the Curve > 0.7

FEDGILEQPPEQ, PFPQKTVEIPEQ, FPLRDAPEQQPE, FPQQPFWLTEQP, FDVFAHPFPFPQ, AWCPADFPEEQP, FPEPAPSQEQPF, AEVSLQEQPPEQ, EMIWNFPFPEQP, EQPPEQAEVSLQ, FPEQPEYGDGVS, PFPPEQALLVEP, HDQNSNQPFQPE, PFPSVDILRQPE, EQPLTQQGFEQP, FPEFPEVVNFES, QPFQPEYNSAHD, DLCREKPEQEQP, EKLVVRPEQQPE, FPQPGYEGWEQP, QPEQPEYQGSSF, PFPNRSLIVQPF, DCTLSLPEQQPE, PFPSVDSLTFPE, DAVEEGQPEPEQ, ASTGYQQPEPFP, FEGRNYFPEFPQ, EQPLQNPLPQPF, GWQALDFPQPFP, PEQRKLVAEFPE, QPEPVPVRAFPQ, PFPQPFVFAEVN, QPFLAERDLFPE, PEQPEQVDQQDC, EQPSGMVNCEQP, FPELCARTVPFP, PFPLLFNAWPFP, HLNKLAPEQQPE, EQPNAPIGLPFP, FPEREAFTREQP, FPQPFPAAVACT, QPFPEQYCCGPV, EQPQSMNMGPFP, CRLLLCPEQPEQ, IPTRVVFPEEQP, QPFLHMGLHQPE, PFPLSLEASQPE, FPQNGRDHHQPF, QPENNTAEEFPE, PFPLDPTPQQPF, AHITNNEQPEQP, FPQKVRMDLQPF, FPEMGSDFDQPF, PEQKSVGRDQPE, IKVRALPFPPEQ, FPENFHCWVPEQ, GRVVSGFPQQPF, QPEPFPASTGYQ, AAVACTFPQPFP, PFPPEQWMTRPD, PEQEQPWVESWM, QPEPVYVGRFPE, PEQNYEASVQPF, EQPQPFVVDWIQ, QPEQPEYPEGSS, PFPPKQKRKQPF, QPFNFGQFEEQP, QPEQPFVNADVV, ALLVEPPFPPEQ, EGDLSTQPFQPF, PEQNCNDDQQPF, PFPTRANHLPEQ, DQGVLLPEQQPE, GPECGTFPQQPF, FPQLVLERCQPF, QPFEQPVVTNYN, GLYRLSQPFEQP, ADAVYLPEQQPF, FPQSEGTYCQPE, FPQSNLLIEPEQ, ENPEIKFPQPFP, QPFQEYVLTFPQ, QPFSWIGSVFPQ, EDITHTEQPQPF, CQRVKYQPEPEQ, EIPDPVFPQQPE, EGAGLTQPEPEQ, QPESFVLGHPEQ, PEQKNHGCQEQP, PFPPQEKSEEQP, QPFPVEAGEFPE, EQPMAEELVFPE, IKIRILPFPPEQ, ILDICLPFPFPQ, FPELTLHFEFPE, DLYLENQPFPEQ, HTYKYPPFPFPQ, EQPFPEVIIGPA, DGSVHKFPEPFP, FPQLEGCTFFPE, QPERCDLELQPF, QPETKARFPQPE, FPQRNEFGEFPE, CWVFAAFPQQPE, FPELAEKEEQPE, QPFPFPWDNNYG, FPQRRSSPVFPE, ESNLIKPEQQPF, DLLPLHEQPFPE, DCLTESQPFPEQ, GHFILLPEQQPE, FSEKSVFPEQPE, QPEEQPTVSYNG, GEEVKVPEQPEQ, EPVINSQPEPEQ, EEERQEEQPQPF, HHTADLQPEQPE, GTKYLLPFPFPE, EQPTFTVEGPFP, GEIQGDQPEQPF, PFPLPVALEFPE, CILYEKEQPFPE, QPFPKFLKNQPE, FPQLTFSVVPEQ, PFPEQPVVTGPA, EKYRDCFPEPEQ, PFPTATVVDQPE, PFPLDVNPKQPF, FPQQGSAKFQPE, PFPRDEREDQPF, EQPEQPVRRGQP, PFPSVPLCIQPE, ILGEPKQPFQPE, FPQPFPVSPMSW, QPELHKLVVQPE, GFIYQGFPQPFP, EQPEQPAHITNN, EEYVCRFPEPFP, PEQPDLQPGQPE, QPFFPQTTPANA, EQPLTEEQKQPE, ESDKLKQPFPEQ, EQPNGILGPEQP, PFPQEAGTKFPQ, EETGMAPFPEQP, PFPMAMRIRQPF, QPELLGRWDQPE, DAPFVFQPFQPF, IEYFRNEQPFPE, QPESTKYDAQPF, CLILLDQPEPFP, FPERCLGIPEQP, EQPNIPWNFPFP, GDKSEMPEQFPE, PFPFPQYLDSEE, FPENSYLLAPEQ, EQPRAIKEGQPF, CTVLRCFPQEQP, PEQKYGQCWFPQ, EGDWTAPEQEQP, PEQQPFADAVYL, PFPLKAVKGEQP, EQPSSEEREPFP, EQPRDCSRRPEQ, EQPNVIIGPFPE, PEQLLNLNLPEQ, EQPSLQLTTFPE, PEQNLEPFSQPF, HKSINRFPEEQP, EQPLRRWKNPEQ, PFPKNAGRDEQP, ELETNGPFPQPF