Serological Investigation of Persistent Villous Atrophy in Celiac Disease

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INTRODUCTION: **Persistent villous atrophy (VA) is not uncommon in celiac disease (CeD) while patients take a gluten-free diet (GFD).**

METHODS: **We conducted a retrospective study with 122 serum samples collected from controls and patients with CeD either at the initial diagnosis or at the follow-up during endoscopy. These samples were assigned to 3 groups: nonceliac control, non-VA CeD (Marsh score 0–2), and VA CeD (Marsh score 3a-3c). We established an in-house multiplex assay to identify potential serological biomarkers for VA. We assessed autoantibodies reported to affect the small intestine, including IgA and IgG antibodies against tissue transglutaminase (tTG), interferons, villin, actin, autoimmune enteropathy-related 75 kDa antigen (AIE-75), and tryptophan hydroxylase (TPH)-1, as well as 27 cytokines. The apolipoproteins quantified included apo A1, apo B-100, and apo A4, which were produced predominantly by the intestinal epithelium or expressed specifically in villi.**

RESULTS: **Autoantibody levels were high only for tTG antibodies, which performed well in initial CeD diagnosis, but suboptimally for VA prediction during follow-up, because 14.6% of the follow-up patients with VA had low tTG-IgA. Increasing dilution improved tTG-IgA quantification, particularly when the antibody levels were extremely high but did not significantly improve VA detection. Among those with low tTG-IgA and persistent VA, high proinflammatory cytokines were observed in 2 patients. Median low-density lipoprotein cholesterol levels were significantly lower in the VA CeD group ($P = 0.03$). Apolipoprotein levels were similar in patients with and without VA but diverged between those on a GFD or not.**

DISCUSSION: **tTG-IgA as a biomarker is suboptimal for VA prediction while on a GFD. Persistent VA is associated with low low-density lipoprotein cholesterol levels and partially related to persistent high proinflammatory cytokines.**

KEYWORDS: celiac disease; autoantibodies; cytokines; villous atrophy; gluten-free diet

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/B17, http://links.lww.com/CTG/B18, http://links.lww.com/CTG/B19, http://links.lww.com/CTG/B20, http://links.lww.com/CTG/B21, http://links.lww.com/CTG/B22, http://links.lww.com/CTG/B23

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INTRODUCTION

The prevalence of celiac disease (CeD) is estimated at about 0.7% globally (1). Chronic diarrhea, constipation, flatulence, abdominal pain, and weight loss are common symptoms of CeD, impairing quality of life (2). If left untreated, iron deficiency anemia, bone density loss, neurological impairment, and even small intestine malignancies may occur (3). The only available treatment for CeD is a gluten-free diet (GFD), but 30%–52% of the patients on such diets continue to report ongoing symptoms (4,5), and 10%–52% have persistent villous atrophy (VA) (6,7). Recent clinical trials have demonstrated the potential efficacy of transglutaminase 2 inhibitors, latiglutenase, and immune

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modulators for CeD treatment (8–10), but none of these agents has yet received regulatory approval (11). By contrast to clinical trials for inflammatory bowel disease, the primary end point for clinical trials on CeD remains to be defined. Histological improvement has been proposed as the primary end point for clinical trials for CeD treatments (8,12) but is not widely accepted as a primary end point for the approval of new medications because of the cost of endoscopy, a patchy distribution of mucosal damage, and inconsistency between pathologists in assessing severity of VA (12).

In recent decades, serological studies have prevailed as the principal method for CeD diagnosis. According to the guidelines of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition, CeD can be diagnosed without biopsy if tTG-IgA levels are more than 10 times higher than the upper limit of the normal range (ULN), provided that endomysial antibody is also detected (13,14). However, commercially available tTG-IgA testing kits, typically based on enzyme-linked immunosorbent assay (ELISA), remain incompletely standardized (15,16). Different tTG-IgA positive sera are used for calibration, and the results are expressed in arbitrary units (17). The ULN for tTG-IgA, like the minimum detection limit for total IgA ELISA quantification, generally ranges from 0.1 to 0.4 ng/mL. As with all ELISAs, there are concerns that low or moderate levels (3× ULN∼10× ULN) may represent false-positive results. If the cut-off value shifts from 1× ULN to 10× ULN, the sensitivity for CeD diagnosis decreases from 72.5%–98.6% (18,19) to 51.9%–70.0% (19,20). The concentration of tTG-IgA decreases in patients on a GFD (21), affecting the ability of this biomarker to predict mucosal recovery. Thus, a normalization of tTG-IgA levels cannot be interpreted as reflecting a healing of the mucosa or improvement of VA (22). Nonetheless, persistent VA could increase the risk of complications, including refractory CeD, malignancy, and mortality (23). Given these disadvantages of current tTG-IgA testing, we conducted this study to search for potential new serological markers for VA in CeD and to improve tTG-IgA quantification through in-house immunoassays.

METHODS

Study design and clinical characteristics of the cohort

We performed a retrospective study to investigate serum biomarkers for VA in CeD. This study was approved by the Institutional Review Board of Columbia University Irving Medical Center (Protocol AAAB2472: "CeD and IBD tissue and cell bank"). We first screened clinical and pathological information of a total of 2096 serum samples collected from 2008 to 2019 in the CeD Center biobank of the Columbia University Irving Medical Center. We selected samples satisfying the following criteria: (i) sample collection on the same day as endoscopic evaluation before the procedure; (ii) pathological evaluation performed and a Marsh score available. We found 122 samples satisfying these criteria, which were then deidentified for further investigation (Figure 1a). These serum samples came from 121 individuals: 46 (38%) male patients and 75 (62%) female patients. Two samples were collected from the single patient at different follow-up visits. The diagnosis of CeD had been confirmed for 106 of these samples: 28 at initial diagnosis and 78 during follow-up visits. Regarding the interval from the initial diagnosis to follow-up, all were collected at least 1 year after the initial diagnosis, with a median interval of 7 years (interquartile range, 2–12 years). Most of the patients received a follow-up endoscopy because of diarrhea (51.3%) or as a routine care (32.1%). Other uncommon indications included persistent anemia (7.7%) and abdominal pain (3.8%). We defined persistent VA for those patients being on GFD for at least 1 year who still have VA on the follow-up endoscopy. All the patients have been guided by a dietician to be compliant to GFD; no urine or stool samples were collected to monitor their GFD compliance. Sixteen samples were obtained from controls without CeD undergoing endoscopy. Marsh scores and clinical characteristics at the time of sampling are summarized in Figure 1b. The patients with confirmed CeD were assigned to the non-VA CeD (Marsh 0–2) group or the VA CeD (Marsh 3a-3c) group.

Quantification of autoantibodies, cytokines, and apolipoproteins

Biomarkers were determined by multiplex particle-based flow cytometry. An in-house multiplex assay was developed to determine total IgA levels and the levels of autoantibodies against IFN-α, IFN-γ, tTG, actin, AIE-75, tryptophan hydroxylase-1 (TPH-1), and villin (see Supplementary Figure 1, http://links.lww.com/CTG/B18). Recombinant human proteins were covalently coupled to carboxylated beads of various sizes (Bio-Plex; Bio-Rad Laboratories) for autoantibody detection. A list of the proteins used is provided in Supplementary Digital Content (see Supplementary Table 1, http://links.lww.com/CTG/B17). Beads were first activated and then incubated with the corresponding proteins at a final concentration of 20 μg/mL in the reaction mixture overnight on a rotator at 4 °C. Serum samples were diluted by 1:100, 1:1,000, and 1:8,000 in 1% bovine serum albumin. Protein-coupled beads were incubated with the diluted serum samples in 96-well filter plates (MultiScreenHTS; Millipore) on a horizontal shaker for 1 hour at room temperature in the dark. The beads from each well were then washed 3 times and split into 2 batches, one of which was incubated for 30 minutes at room temperature with biotin-labeled anti-human IgG-Fc antibody (1:10,000, Leinco), the other batch being incubated under the same condition with anti-human IgA antibody (1:5,000, IgA5-3B, Abcam, ab53270). Streptavidin/PE was added at a final concentration of 2 μg/mL, and the mixture was incubated for 10 minutes at room temperature. The beads were then subjected to fluorescence-activated cell sorting (Attune NxT Flow Cytometer, Thermo Fisher Scientific), and FlowJo 10.8.1 software (FlowJo LLC) was adopted to analyze the data. A standard curve was generated, and median fluorescence intensity (MFI) was determined for further quantification.

Similarly, we measured the level of cytokines using a commercially available kit, Bio-Plex Pro Human Cytokine 27-plex assay (BioRad), and determined the level of apolipoproteins with the Legendplex Human Apolipoprotein (Apo) Panel (11-plex) kit (BioLegend, 740453). Apo A4 levels were successfully established with 2 sets of anti-apo A4 antibodies free from bovine serum albumin and azide: ab242651 as the capture antibody and ab242896 as the detector antibody (Abcam). A biotinylation kit (Abcam, ab201795) was used to label the detector antibody with biotin. Recombinant human apolipoprotein A-IV/apo A4 protein (R&D Systems, 9606-AP-050) was used to generate a standard curve. Performers of the assays were blinded from the diagnoses and mucosal status of the patients.

Statistical analyses

All data analyses were performed with R statistical software (R version 3.6.3). Statistical significance was defined as $P < 0.05$ for all analyses. The false discovery rate method was used to correct for multiple comparisons. Continuous data are presented as the

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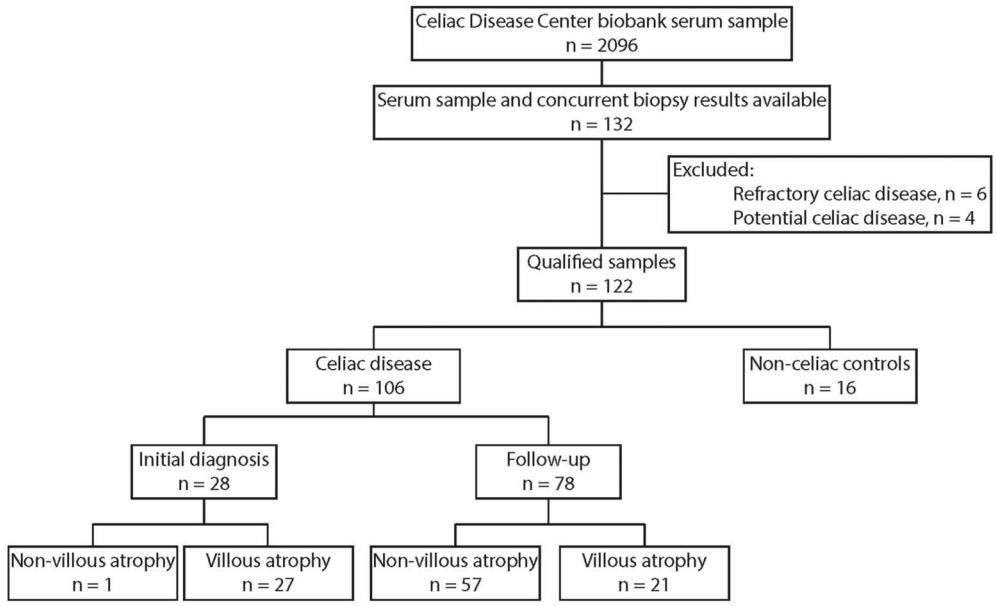

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Characteristics	Non-celiac control (n = 16)	CeD					
		Marsh 0 (n = 27)	Marsh 1 (n = 29)	Marsh 2 (n = 2)	Marsh 3a (n = 24)	Marsh 3b (n = 13)	Marsh 3c (n =11)
Gender							
Male	7 (43.8%)	9 (33.3%)	12 (41.4%)	1 (50.0%)	4 (16.7%)	9 (69.2%)	5 (45.5%)
Female	9 (56.3%)	18 (66.7%)	17 (58.6%)	1 (50.0%)	20 (83.3%)	4 (30.8%)	6 (54.5%)
Age (years) #	53.0 (43.9-66.1)	42.0 (22.9-55.3)	57.1 (42.3-70.1)	45.9 (35.5-56.3)	52.9 (30.7-63.4)	35.7 (26.6-46.3)	29.8 (20.4-42.7)
Initial diagnosis	N/A	0	1 (3.4%)	0	9 (37.5%)	8 (61.5%)	10 (90.9%)
GFD	2 (14.3%*)	27 (100.0%)	27 (93.1%)	2 (100.0%)	16 (66.7%)	5 (38.5%)	2 (18.2%)

Figure 1. Clinical characteristics of the study cohort. (**a**) Flowchart of sample selection and a summary of qualified samples. (**b**) Clinical characteristics of patients with CeD and nonceliac controls with different Marsh scores. #Age is shown in median and 25th to 75th percentile range. *In the nonceliac control group, data on GFD were not available for 2 samples. CeD, celiac disease; GFD, gluten-free diet.

median and interquartile range. Because the MFI data did not follow a normal distribution, Mann-Whitney *U* tests were conducted for comparisons between 2 groups and the Kruskal-Wallis tests for comparisons between 3 or more groups. χ^2 tests were performed for the analysis of categorical data. Heatmap and cluster analyses were conducted with the pheatmap package (version 1.0.12). Receiver operating characteristic curve analysis was adopted to determine the sensitivity and specificity of candidate tTG autoantibody determinations in different patient subgroups. The optimal cut-off values were determined by calculating the Youden index, and the Delong test was used to compare area under the curve (AUC) values (24). Missing data were excluded from the analysis of the specific item.

RESULTS

An in-house tTG-IgA and IgA assay for investigating VA in CeD

An in-house multiplex assay was established to better determine total IgA and tTG-IgA levels. As shown by the standard curves (see Supplementary Figure 2, http://links.lww.com/CTG/B19), total IgA detection could be quantified within the range extending from 0.16 ng/mL to 10 μg/mL. When an arbitrary ULN was set at an MFI of 400, our multiplex assay can measure serum antibodies ranging from 2 to 125,000 arbitrary units. One sample from a patient with selective IgA deficiency and another with relatively low IgA levels confirmed the accuracy of our measurements (see Supplementary Figure 3, http://links.lww.com/CTG/B20). For tTG-IgA determination with a standard 1:100 dilution, 16 patients (33.3%) with VA were found to have tTG-IgA levels above the maximum limit of detection, as compared with only one (1.7%) in patients without VA. In addition, 12 (75.0%) nonceliac controls had tTG-IgA levels above the ULN, suggesting nonspecific binding at 1:100 dilution. For those with extremely high tTG-IgA titers, differentiation was improved by a dilution of 1:1,000 or 1:8,000, which also yielded a lower background signal (Figure 2a–c and see Supplementary Figure 4A, http://links.lww.com/CTG/B21). The sensitivity and specificity of tTG-IgA for the initial diagnosis of CeD were similar with an AUC above 0.99 at all 3 dilutions (see Supplementary Table 2, http://links.lww.com/CTG/B17). Significantly lower tTG-IgA levels were found in patients on a GFD or during the follow-up (see Supplementary

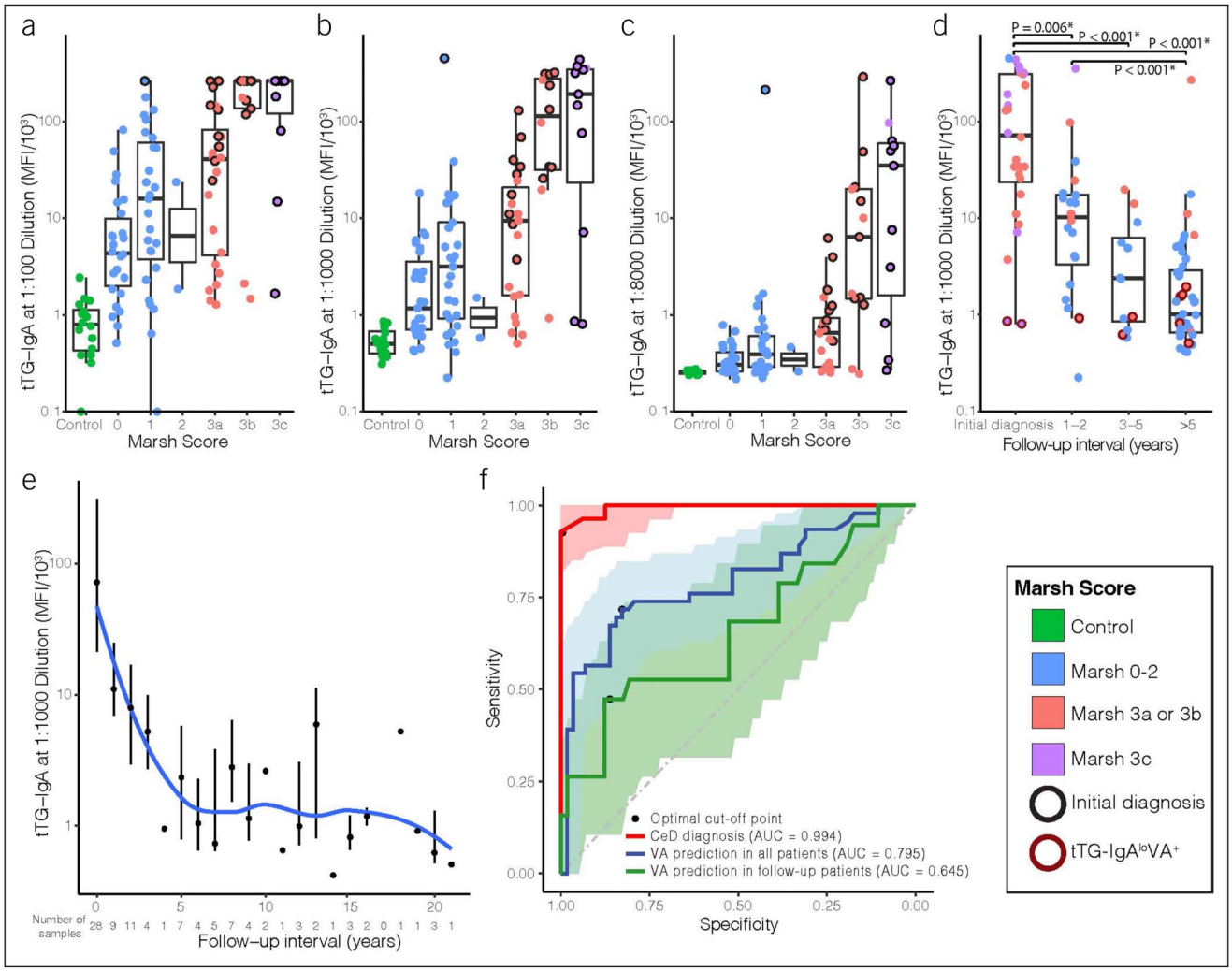

Figure 2. Analyses of antitissue transglutaminase IgA antibody (tTG-IgA) levels. (**a–c**) tTG-IgA determination in serum samples from nonceliac controls and patients with CeD at various dilutions, according to Marsh score. (**d–e**). Changes in tTG-IgA levels in patients with CeD over the course of GFD. (**f**) Receiver operating characteristic (ROC) curve for tTG-IgA levels at a 1:1,000 dilution, for the initial diagnosis of CeD, and the prediction of villous atrophy (VA). CeD, celiac disease; GFD, gluten-free diet.

Figure 5, http://links.lww.com/CTG/B22). tTG-IgA levels decreased by about 85.7% after initiating GFD for 1 year and continued decreasing to a lower level until 5 years on GFD (Figure 2d–e). Consistent with current recommendations (25), the patient with selective IgA deficiency in our study had a high tTG-IgG titer (see Supplementary Figures 4B–4D, http://links.lww.com/CTG/B21). However, we found no correlation between tTG-IgA and tTG-IgG levels (see Supplementary Figures 4B–4D, http://links.lww.com/CTG/B21). Caution is therefore required in the interpretation of positive tTG-IgG results. In conclusion, this in-house tTG-IgA and total IgA assay had a high sensitivity and specificity for the initial diagnosis of CeD. It takes about 5 years for tTG-IgA level to be normalized for most of the patients with CeD on GFD.

Prediction of villous atrophy with tTG autoantibodies in patients with CeD

We then investigated the association between tTG-IgA and VA. For the total cohort of patients with CeD, at either initial diagnosis or follow-up, tTG-IgA levels were reasonably predictive of VA, as shown in Supplementary Digital Content (Supplementary Table 2, http://links.lww.com/CTG/B17), with the highest AUC of 0.795 obtained at a dilution of 1:1,000. However, the AUC decreased to about 0.6 during the follow-up of patients with CeD. tTG-IgA performed better for initial CeD diagnosis than for the prediction of VA during follow-up (Figure 2f). We found that 12 of the 48 patients with VA had low tTG-IgA levels (Figure 3a), accounting for the poor performance of tTG-IgA for predicting VA during CeD follow-up. To gain further insights into the cause for VA in patients with low tTG-IgA levels, we investigated this group of patients further, by comparing patients with VA but low tTG-IgA (tTG-IgA^{lo} VA^{+}) and patients with VA and high tTG-IgA (tTG-IgA^{hi} VA^{+}). We found that tTG-IgA^{lo} VA^{+} patients tended to be older than those with higher levels, and they were more likely to be female, on a GFD, and to have partial VA (Figure 3a).

We then investigated cytokine levels in these tTG-IgA^{lo} VA^{+} patients, as well as in nonceliac controls and tTG-IgA^{hi} VA^{+} patients. No significant difference in cytokine levels was observed

a

Characteristics	tTG-IgA^{lo} VA^{+} (n = 12)	tTG-IgA^{hi} VA^{+} (n = 16)	P value
Gender			0.127
Male	4 (33.3%)	10 (62.5%)	
Female	8 (66.7%)	6 (37.5%)	
Age	56.8 (42.8-65.3)	34.6 (22.1-48.4)	0.023*
Initial diagnosis	2 (16.7%)	13 (81.3%)	0.001*
Gluten-free diet	10 (83.3%)	4 (25.0%)	0.004*
Positive serology	0	16 (100.0%)	< 0.001*
Marsh score			0.013*
3a	8 (66.7%)	2 (12.5%)	
3b	2 (16.7%)	7 (43.8%)	
3c	2 (16.7%)	7 (43.8%)	

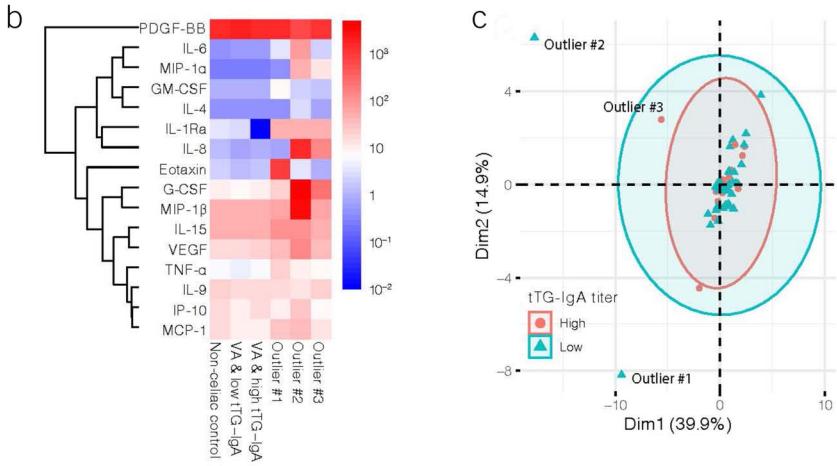

Figure 3. Exploration of the causes for persistent villous atrophy in patients with low antitissue transglutaminase IgA antibody (tTG-IgA) levels. (**a**) General characteristics of patients with a Marsh score of 3 but low tTG-IgA levels (tTG-IgA^{lo} VA^{+}) and patients with a Marsh score of 3 and high tTG-IgA levels (tTG-IgA^{hi} VA^{+}). Quantification of tTG-IgA was expressed in 10^3 median fluorescence intensities (MFI). tTG-IgA^{hi} VA^{+} was defined as tTG-IgA above detection limit at 1:100 dilution, and tTG-IgA^{lo} VA^{+} was defined as tTG-IgA below upper limit of normal (MFI 400) at 1:8,000 dilution. Data are shown in median and 25th to 75th percentile range. (**b**) Heatmap of cytokine levels (pg/mL) in 3 major groups and 3 outliers. The 3 groups are nonceliac controls, tTG-IgA^{lo} VA^{+} patients, and tTG-IgA^{hi} VA^{+} patients, with outliers excluded from corresponding groups. (**c**) Results of principle component analysis, with all 27 cytokines included and grouped by tTG-IgA levels. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; IP-10, interferon γ-induced protein-10; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

between 3 above groups (see Supplementary Figure 6, http://links.lww.com/CTG/B23). Cluster analysis by the complete linkage method identified 2 major clusters and 3 outliers (Figure 3b), which were confirmed by principal component analysis (Figure 3C), but these clusters did not differ in diet, Marsh score, or tTG-IgA titer. However, 2 of the 3 outliers both had persistent VA and low tTG-IgA levels, and they exhibited high proinflammatory cytokine levels (especially IL-6 and IL-15). In conclusion, patients on GFD tend to have low tTG-IgA levels, and about 14.5% of follow-up patients on GFD have a low tTG-IgA level but nevertheless present VA on biopsy, which could be partially attributed to high level of proinflammatory cytokines.

Autoantibodies against interferons, AIE-75, actin, and villin in CeD

We then investigated the possible involvement of other autoantibodies in VA. We determined the levels of IgA and IgG antibodies against interferons, which are frequently present in autoimmune diseases and severe COVID-19 (26,27); anti-AIE-75 antibodies (directed against a protein also known as USH1C or harmonin), implicated in autoimmune enteropathy (28,29); and antibodies against TPH-1, villin, and actin, implicated in immune-mediated damage to the small intestine (28–30). None of the patients with CeD or controls had high titers (>10× ULN) of antibodies against the IFNs tested (Figure 4a,b). In a few samples, antivillin IgG (n = 17) or anti-actin IgG (n = 18) antibody levels were higher than 3 × ULN, with no significant differences between the 3 groups (see Supplementary Table 3, http://links.lww.com/CTG/B17). Two AIE-75 reagents were used in this study, and stronger signals were obtained from almost all samples with the one produced by *Escherichia coli* (Sigma), suggesting nonspecific binding (Figure 4c,d). The cluster analysis of all IgA autoantibodies yielded 2 major subgroups, clusters 1 and 2 (Figure 4c). The frequency of VA was significantly lower (21.2% vs 80.6%, $P < 0.001$), but the percentage of patients on a GFD was significantly higher (79.5% vs 38.9%, $P < 0.001$) in cluster 1 than in cluster 2. However, no significant difference was found between clusters identified in the analysis for IgG antibodies, in either VA or GFD (Figure 4d). In

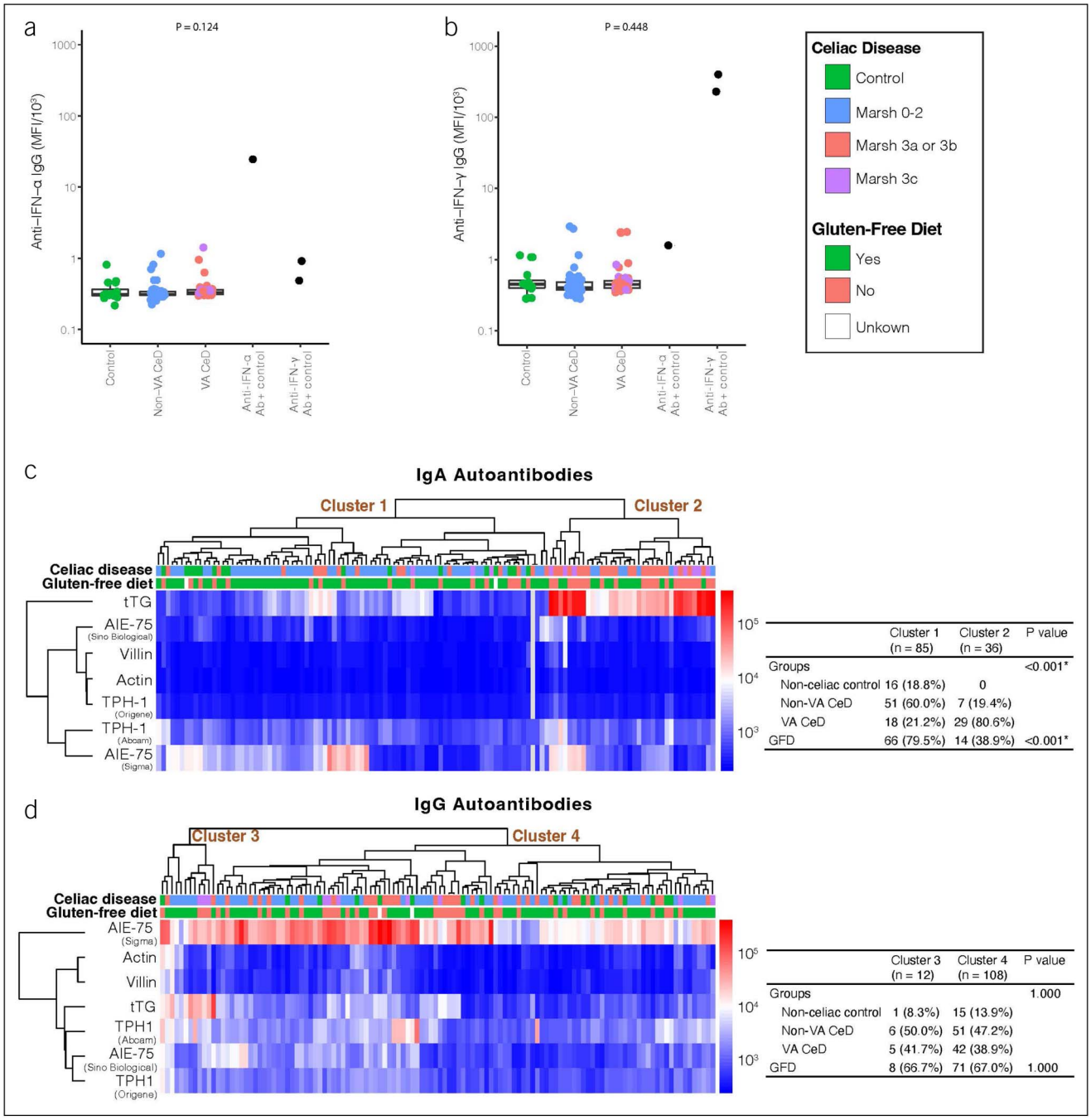

Figure 4. Autoantibody determinations in the nonceliac control, non-VA CeD, and VA CeD groups. (**a**) Anti–IFN-α IgG antibody, 1:8,000 dilution. (**b**) Anti–IFN-γ IgG antibody, 1:8,000 dilution. (**c**) Heatmap of IgA autoantibody levels. (**d**) Heatmap of IgG autoantibody levels. Ab, antibody; AIE-75, autoimmune enteropathy-related 75 kDa antigen; IFN, interferon; MFI, median fluorescence intensity; TPH1, tryptophan hydroxylase 1; tTG, tissue transglutaminase; VA, villous atrophy.

conclusion, none of the autoantibodies tested, with the exception of tTG-IgA, was associated with VA in CeD.

Dysregulation of cholesterol metabolism and apolipoproteins in CeD

The small intestine plays an essential role in cholesterol metabolism (31). We and others have shown that total cholesterol levels increase in patients with CeD on a GFD (32,33). We found no significant difference between the nonceliac control, non-VA CeD, and VA CeD groups in total cholesterol (Figure 5a), triglyceride (Figure 5b), or high-density lipoprotein cholesterol levels (Figure 5c), irrespective of GFD. However, low-density lipoprotein cholesterol levels were significantly lower in the VA CeD group ($P = 0.030$; Figure 5d). Apolipoproteins are involved in lipid absorption and transportation, and the small intestine is a major source of apolipoproteins apoA1, A4, and B (34,35). Apo A4 staining has also been identified as a marker of the transition between villi and crypts (36). However, no significant difference

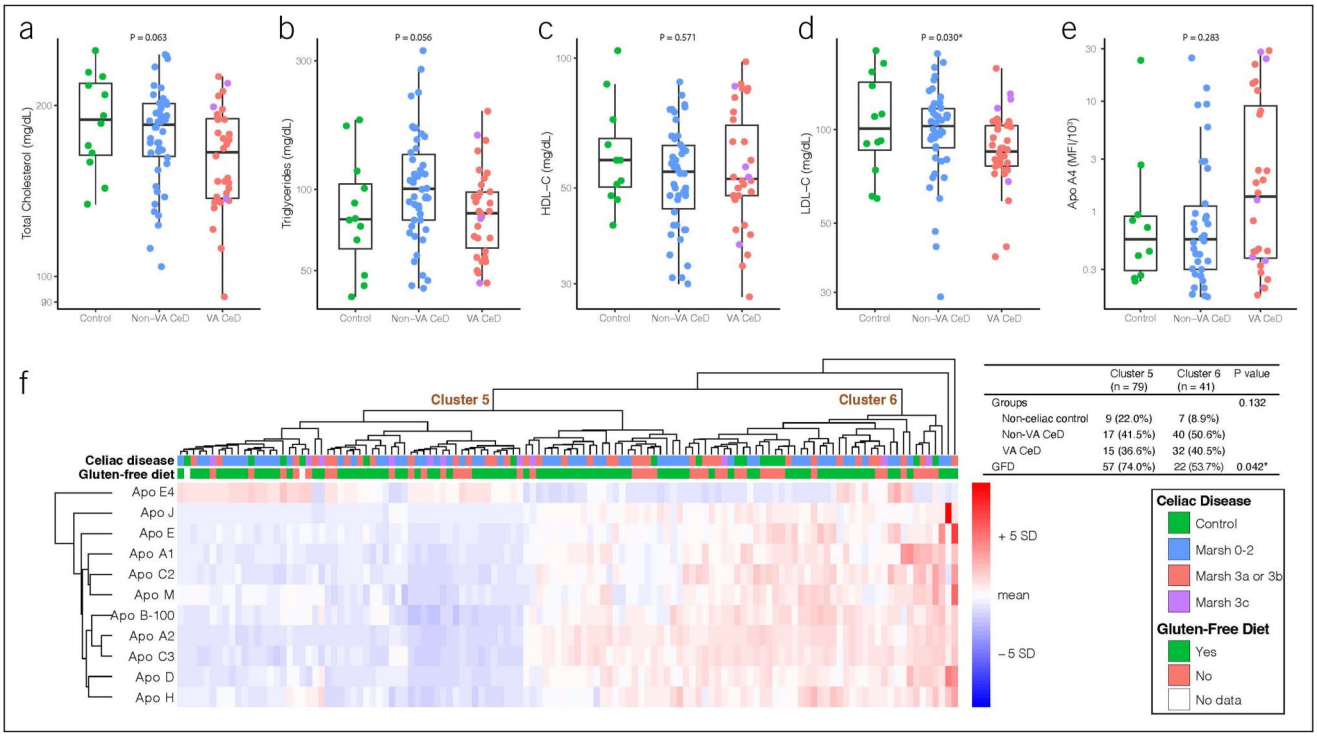

Figure 5. Determination of a panel of lipids, including total cholesterol (**a**), triglycerides (**b**), low-density lipoprotein cholesterol (LDL-C, **c**), high-density lipoprotein cholesterol (HDL-C, **d**), and apo A4 (**e**) in the nonceliac control, non-VA CeD, and VA CeD groups. (**f**) Heatmap and cluster analysis for apolipoproteins. MFI, median fluorescence intensity; VA, villous atrophy.

in apolipoproteins was found between the 3 groups (Figure 5e and see Supplementary Table 4, http://links.lww.com/CTG/B17). Cluster analysis based on apolipoprotein levels identified 2 major subgroups: cluster 5 ($n = 79$) and cluster 6 ($n = 41$; Figure 5f). These 2 clusters did not differ in Marsh score but interestingly, more patients in cluster 5 were on a GFD (74.0% vs 53.7%; $P = 0.042$; Figure 5f), suggesting that a GFD may affect the homeostasis of serum apolipoproteins. Further analysis revealed that the patients on a GFD had significantly higher serum apo A2, apo C2, apo D, apo J, and apo M concentrations but lower serum apo C3, apo E, apo E4, apo H, and apo A4 concentrations (see Supplementary Table 5, http://links.lww.com/CTG/B17). VA in CeD was, thus, associated with mild changes in lipid metabolism.

DISCUSSION

Clinicians have focused on malabsorption and VA for monitoring patients with CeD and assessing the response to a GFD. However, the evaluation of VA requires a biopsy of the small intestine, which can be difficult, although the staining of apo A4, an apolipoprotein originating from the small intestinal villus (37), has recently given encouraging results for the measurement of villus atrophy (36,38). Could serum lipid and apolipoprotein levels serve as a marker for VA? We found that patients with CeD with VA had a lower median low-density lipoprotein cholesterol concentration. Previous reports also reported that patients with active CeD had low total cholesterol, high-density lipoprotein cholesterol, and apo A1 levels (39,40), which improved after the introduction of a GFD (32,33). Our results suggest that a spectrum of apolipoproteins may be affected by a GFD. However, those changes were mild, and we observed no significant correlation between apolipoprotein levels and VA. Assessments of apolipoproteins and lipids are not sufficient for the prediction of VA in CeD.

Consistent with previous studies (41), we found that the performance of tTG-IgA determination was excellent for the initial diagnosis of CeD (AUC > 0.99) and that tTG-IgA levels were well correlated with histological changes before GFD (16,42). However, this and other studies have shown that the sensitivity of tTG-IgA for predicting VA decreases considerably after the introduction of a GFD (43). Our findings highlight the lack of utility of tTG-IgG antibody level determinations for diagnosing CeD when total IgA level is in the normal range. Absah et al (44) showed that only 3% of individuals with high tTG-IgG levels, negative for tTG-IgA, and with normal total IgA levels had CeD on biopsy.

We observed no significant production of autoantibodies against interferons, AIE-75, villin, or any other autoantigens. However, in patients with a Marsh score of 3, we observed a striking difference in tTG-IgA levels revealed only when the serum was diluted 1:8,000 and a unique CeD phenotype in patients with low tTG-IgA levels and VA. About 1 in 4 of the patients with CeD in our VA CeD group were presented with low tTG-IgA levels. It has been shown in mouse models that tTG reactive mucosal B-cell proliferation results from gluten digestion with the help of T cells (45). CeD-specific tTG-IgA binds to the N-terminal fragment of tTG, but not the C-terminal fragment, maintaining enzymatic activity (46). Blocking the C-terminal part of tTG has been reported to improve the performance of tTG-IgA determination for predicting VA (46). One recent study generated neoepitopes with 6 amino acids in tTG (aa245-250) shuffled with 6 amino acids from the gliadin peptide. These 12-mer were reported to improve antibody detection and the prediction of mucosal healing in CeD (47). However, none of them addressed

the mechanism of persistent tissue damage in patients with CeD on GFD with low tTG-IgA levels.

This phenomenon of persistent VA in patients with CeD with low tTG-IgA levels on a GFD is fascinating. Although cytokine quantification provided explanation in some of the patients, more investigations are still needed to reveal the underlying cause of VA in others. One of the potential mechanisms might be related to tissue-resident T cells, which can persist throughout the individual's life (48,49). However, because plasma cells were also frequently observed in biopsy specimens from such patients with CeD, further studies are required to identify the driver of these intestinal plasma cells and their pathologic role in sustaining mucosal inflammation. A combination of single-cell sequencing and epitope mapping might provide more answers. Our results highlight the lack of serological markers for the assessment of VA in patients with CeD on a GFD and the need for patients to undergo endoscopic biopsy to assess healing of the small bowel mucosa.

CONFLICTS OF INTEREST

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Study Highlights

WHAT IS KNOWN

- ✓ IgA antitissue transglutaminase antibody (tTG-IgA) performs well in the diagnosis of celiac disease (CeD).
- ✓ tTG-IgA cannot predict villous atrophy (VA) during follow-up.

WHAT IS NEW HERE

- ✓ A better quantification of tTG-IgA levels could be achieved by increasing serum dilution but remained suboptimal in predicting VA during CeD follow-up.
- ✓ Proinflammation may be related to persistent VA in a proportion of patients with CeD on gluten-free diet.
- ✓ Other autoantibodies commonly seen in autoimmune gastrointestinal disorders were not related to CeD.

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