SUPPLEMENTARTY FILE

Subclass Profile of IgG Antibody Response to Gluten Differentiates Non-Celiac Gluten Sensitivity from Celiac Disease

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Supplementary Methods

Patients and controls. The study included 80 individuals with non-celiac gluten sensitivity (NCGS) who met the diagnostic criteria proposed by an expert group [1] and who were identified using a previously described structured symptom questionnaire [2, 3] (a modified version of the Gastrointestinal Symptom Rating Scale (GSRS) designed to rate symptoms commonly associated with NCGS). All NCGS subjects reported experiencing gastrointestinal and/or extra-intestinal symptoms after ingestion of gluten-containing foods, including wheat, rye, or barley. The most common gastrointestinal symptoms included bloating, abdominal pain, diarrhea, nausea, and heartburn, while the most prominent extra-intestinal symptoms were fatigue, headache, anxiety, cognitive difficulties, and numbness in arms and legs. The reported symptoms in all subjects improved or disappeared when those foods were withdrawn for a period of 6 months, and recurred when they were re-introduced for a period of up to 1 month. Individuals were excluded if they were already on a restrictive diet or had used nonsteroidal anti-inflammatory drugs within the past 6 months, if they were positive for the celiac disease-specific intestinal histologic findings or the IgA anti-endomysial or -transglutaminase 2 (TG2) autoantibodies, or if they were positive for wheat allergy-specific IgE serology or skin prick test. A history of autoimmunity or autoantibody reactivity was present in 20 NCGS patients (25%), represented mainly by Hashimoto's thyroiditis and by antinuclear antibody positivity (demonstrated by indirect immunofluorescence on HEp-2 cells), similar to previously published data [4, 5]. In addition, 49 patients (61.2%) met criteria for irritable bowel syndrome and 63 (78.8%) for functional dyspepsia according to Rome IV [6, 7]. All patients underwent an esophagogastroduodenoscopy with gastric biopsy to rule out *H. pylori* infection. A total of 6 intestinal biopsies, including 2 from the duodenal bulb and 4 from the distal duodenum, were

taken from each individual. The study also included 40 patients with biopsy-proven celiac disease (CD) and 40 healthy subjects, recruited as part of the same protocol that included the NCGS individuals. All cases of CD were positive for IgA anti-endomysial and IgA anti-transglutaminase 2 (TG2) autoantibodies, biopsy-proven, and diagnosed according to established criteria [8]. Rome IV [6, 9], GSRS [10], and SF-36 Health Survey [11] were utilized to evaluate the general health of unaffected controls. Individuals who had a history of liver disease, liver function blood test results (total protein, aspartate transaminase, alanine transaminase, alkaline phosphatase, albumin, globulin, and bilirubin) outside of normal range, or a recent infection were excluded from all cohorts in the study. Cases of IgA deficiency and IgG subclass deficiency were excluded from all cohorts. Inflammatory bowel disease was ruled out in all cases.

All samples were collected at time of study entry while participants were on an unrestricted (gluten-containing) diet with written informed consent under institutional review board-approved protocols at St. Orsola-Malpighi Hospital, Bologna, Italy. Serum specimens were kept at -80 °C to maintain stability. This study was approved by the Institutional Review Board of Columbia University Medical Center.

Assays. Serum levels of total IgG reactivity to gluten and individual IgG subclass reactivities to gluten were measured separately by an enzyme-linked immunosorbent assay (ELISA), similarly to our prior studies [12, 13]. The antigen used for the assays was the Prolamine Working Group (PWG) reference gliadin, as previously described and characterized [12, 14]. A 2 mg/mL stock solution of the PWG gliadin was prepared in 70% ethanol. Wells of 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 µL/well of a 0.01 mg/mL solution of protein in 0.1 M carbonate buffer (pH 9.6) or left uncoated to serve as controls. After incubation at 37°C for 1 h, wells were washed and blocked by incubation with

1% bovine serum albumin (BSA) in PBS containing 0.05% Tween-20 (PBST) for 1.5 h at room temperature. Serum samples were diluted at 1:300, added at 50 µL/well in duplicates, and incubated for 1 h. Each plate contained a positive control sample with a high level of relevant IgG subclass reactivity to gluten, as determined in a preliminary screen. After washing, the wells were incubated with HRP-conjugated anti-human IgG1 (Life Technologies), IgG2 (Life Technologies), IgG3 (Life Technologies), or IgG4 (Southern Biotech) secondary antibodies for 50 min. Plates were washed and 50 µL of developing solution, containing 27 mM citric acid, 50 mM Na_2HPO_4, 5.5 mM *o*-phenylenediamine, and 0.01% H_2O_2 (pH 5), was added to each well. Absorbance was measured at 450 nm after 20 min. All samples were tested in duplicate. Absorbance values were corrected for non-specific binding by subtraction of the mean absorbance of the associated BSA-coated control wells. The corrected values were first normalized according to the mean value of the positive control duplicate on each plate. The mean antibody level for the healthy control cohort was then set as 1.0 AU and all other results were normalized to this value. The cutoff value for anti-gliadin IgG positivity was assigned as two standard deviations above the mean for the healthy control group.

Serum levels of intestinal fatty acid-binding protein (FABP2) were measured as we have previously described [13]. FABP2 is a cytosolic protein specific to intestinal epithelial cells that is released into systemic circulation upon cellular damage.[15] Increased concentrations of circulating FABP2 reflect epithelial cell loss and enhanced enterocyte turnover rate [15-17] and have been associated with both CD and NCGS [13]. IgA antibody to recombinant human TG2, a sensitive and specific serologic marker for CD [18], was measured in all serum samples as previously outlined [13]. HLA genotyping to assess CD genetic predisposition was done as shown before [12].

Data analysis. Group differences were analyzed by the Kruskal-Wallis one-way analysis of variance with post-hoc testing. Correction for multiple comparisons was done using Dunn's statistical hypothesis testing and the multiplicity-adjusted P values are reported for each comparison. Correlation analysis was performed using Spearman's *r*. A multivariate principal component analysis (PCA) was carried out on the entire IgG subclass dataset to assess clustering. All *P* values were 2-sided, and differences were considered statistically significant at $P<0.05$. Statistical analyses were performed with Prism 8 (GraphPad) and Minitab 19 (Minitab).

Supplementary Table 1. Demographic and clinical characteristics of study cohorts.

Subject group	Number of subjects	Mean age—years [SD]	Female sex—no. (%)	Celiac disease-associated HLA DQ2 and/or DQ8—no. (%)	Intestinal biopsy histologic grade: Marsh 0; Marsh 1; Marsh 3—no. (%)
NCGS	80	34.6 [10.3] [a]	62 (78) [a]	21 (26) [b]	48 (60); 32 (40); 0 [b]
Celiac Disease	40	34.5 [13.7] [a]	30 (75) [a]	40 (100) [b]	0, 0, 40 (100) [b]
Healthy	40	35.0 [12.8] [a]	30 (75) [a]	-	-

[a] No statistically significant differences exist between NCGS, celiac disease, and healthy cohorts.

[b] Statistically significant differences exist between the celiac disease and NCGS cohorts ($P<0.0001$ for all comparisons).

References for Supplementary File

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