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

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Brief Communication

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

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Keywords: celiac disease; gluten sensitivity; immune activation; B cell

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FABP2, intestinal fatty acid-binding protein; FcγR, Fc-gamma receptor; HLA, human leukocyte antigen; IFNγ, interferon gamma; NCGS, non-celiac gluten/wheat sensitivity.

Word count: 1,000

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INTRODUCTION

Celiac disease (CD) is an autoimmune enteropathy triggered by exposure to gluten proteins, leading to intestinal inflammation and villous atrophy in genetically predisposed individuals. It is associated with robust B cell and antibody responses to gluten and to the transglutaminase 2 (TG2) autoantigen [1]. In contrast, non-celiac gluten sensitivity (NCGS) is a poorly understood clinical entity defined by onset of symptoms in response to ingestion of gluten-containing food without the prerequisite serologic or histologic features of CD [2]. There are no established biomarkers yet for NCGS, but recent research points to a biological basis, revealing a state of systemic immune activation in conjunction with a compromised intestinal epithelium [2, 3].

We and others have demonstrated a significant increase in IgG antibody to gluten in NCGS at levels similar to CD [2, 3]. Accordingly, it has been speculated that an enhanced IgG response to gluten may be a common link between CD and NCGS [2]. However, whether and how B cell reactivity to gluten may differ in these conditions, especially in the context of possible relevance to intestinal pathology, have not been examined.

In this study, we extend earlier data to show that the anti-gluten IgG antibody in NCGS is significantly different from CD in subclass distribution and in its relationship to intestinal cell damage. The findings are suggestive of a sustained primary B cell response to gluten in CD despite the condition's chronicity, and a more advanced and tolerogenic immune response to gluten in NCGS.

METHODS

Detailed methods are available in Supplementary Methods.

RESULTS

Demographic and clinical characteristics of study cohorts are included in Supplementary Table 1.

The anti-gliadin IgG response in CD patients was comprised primarily of IgG1 and IgG3, which were significantly increased in comparison with the healthy and NCGS cohorts (Fig. 1A,1C). There was a modest elevation in anti-gliadin IgG2 compared with the healthy group and no comparative increase in the IgG4 subclass (Fig. 1B,1D). Within the NCGS cohort, however, the lower contributions of anti-gliadin IgG1 and IgG3 in comparison with CD was compensated by significantly elevated IgG4 (compared with CD and healthy cohorts) and IgG2 (compared with healthy cohort) (Fig. 1A-D). No significant association was detected in this cohort between any anti-gliadin IgG subclass and the Marsh type, HLA-DQ2/DQ8 status, or eligibility for irritable bowel syndrome or functional dyspepsia diagnostic criteria.

The score plot for the principal component analysis of the IgG subclass data demonstrated clustering of the CD and NCGS subjects into discernible groups, further demonstrating the contrasting subclass distributions and suggesting potential biomarker value in these data (Fig. 1E).

Serum concentrations of intestinal fatty acid-binding protein (FABP2), a specific marker of intestinal epithelial cell damage [4], were similarly elevated in the CD and NCGS groups in comparison with healthy cohort ($P<0.0001$ for each) [3]. Within the CD group, only the anti-gliadin IgG3 correlated with FABP2 (Fig. 1H). This correlation was similar in strength to that between anti-gliadin IgG3 and anti-TG2 IgA ($r=0.505$, $P=0.001$). In contrast, FABP2 levels in the NCGS group correlated with anti-gliadin IgG4 and weakly with IgG1 (Fig. 1M,1J).

DISCUSSION

The observed contrast in the IgG subclass distribution and relationship with FABP2 release in NCGS *versus* CD are likely reflective of differences in the evolution and disease relevance of B cell immune responses in the two conditions. Among IgG subclasses, IgG1 and IgG3 are the most potent activators of complement and efficient at binding a wide range of FcγRs [5]. In contrast, IgG2 antibodies generally require higher epitope densities for complement activation and display limited binding to FcγRs [5]. IgG4 antibodies contain structural properties that further distinguish them from other immunoglobulin isotypes and IgG subclasses. They bind weakly to Fc receptors and to complement, and are inefficient at crosslinking of antigens or forming immune complexes [5]. IgG4 has also been shown to induce an anti-inflammatory M2-like macrophage phenotype through inhibition of IFNγ signaling [6]. Considering these properties, the observed increase in the gluten-reactive IgG2 and IgG4 subclasses and the correlation between the IgG4 subclass and FABP2 in NCGS may point to a protective response aimed at dampening the inflammatory effect of other antibodies and immune cells. It is intriguing that these antibody responses are largely absent in CD, where there is instead a correlation between the IgG3 and FABP2.

The evolution in subclass switching of the IgG response to an antigen follows a 1-way direction from IgG3 to IgG1, IgG2, and IgG4 over time. Once a B cell has switched to a downstream subclass, it does not return to a preceding one [7]. It has been suggested that IgG2 and IgG4 are part of the immunologic memory towards harmless and recurring antigens—an advanced immune response stimulated by a more extensive antigen exposure [8]. In addition, the variable regions of IgG2 and IgG4 usually display greater levels of somatic hypermutation than IgG1 or IgG3, which can result in higher affinity for target antigens [7]. As such, the prominence

of the IgG3 subclass and its relationship with the autoimmune response and intestinal cell turnover in CD is suggestive of repeated activation of gluten-specific naive B cells, rather than of memory cells, in response to gluten exposure, despite the chronic nature of the disease. Pathways involved in this phenomenon may represent a source of molecular targets for therapeutic intervention. Possible shortfalls of this study include the lack of other disease controls and the fact that these observational data cannot establish a causal connection between subclass differences and the disease process.

These data warrant further examination of the evolution of gluten-reactive B cell response and subclass switching in CD and NCGS. In addition, information on other aspects of B cell and antibody variability, including affinity, glycosylation profile, and epitope specificity, is expected to contribute to a greater understanding of differences in the immune response to gluten and its relationship with disease pathophysiology in the two conditions. In conjunction with other previously identified markers, these components of the immune response to gluten are expected to provide additional biomarkers that may be informative in the context of stratifying potential disease subsets with varying mechanisms, prognoses, and responses to therapy.

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FIGURE LEGEND

Figure 1. Distribution of IgG subclass antibody reactivity to wheat gluten and relationship with intestinal epithelial cell damage. **A-D)** Log of serum levels of IgG1 (A), IgG2 (B), IgG3 (C), and IgG4 (D) antibody to Prolamine Working Group (PWG) gliadin in cohorts of healthy controls and IgG anti-gliadin-positive celiac disease (CD) and non-celiac gluten sensitivity (NCGS) patients, as determined by ELISA. Horizontal red lines indicate the median for each cohort. **E)** Principal component analysis score plot of the entire anti-gliadin IgG subclass dataset (IgG1, IgG2, IgG3, and IgG4) for CD (red) and NCGS (green) patients. Subjects are plotted in three dimensions using the first through third principal components (PC1, PC2, and PC3). **F-M)** Relationship between FABP2 expression and IgG subclass antibody reactivity to gluten in CD and NCGS patients. Serum FABP2 concentrations in CD patients correlated with levels of anti-gliadin IgG3 antibody (H). In contrast, the NCGS cohort was characterized by a correlation between the levels of anti-gliadin IgG4 antibody and FABP2 concentration (M) and a weaker correlation between anti-gliadin IgG1 antibody and FABP2 (J).

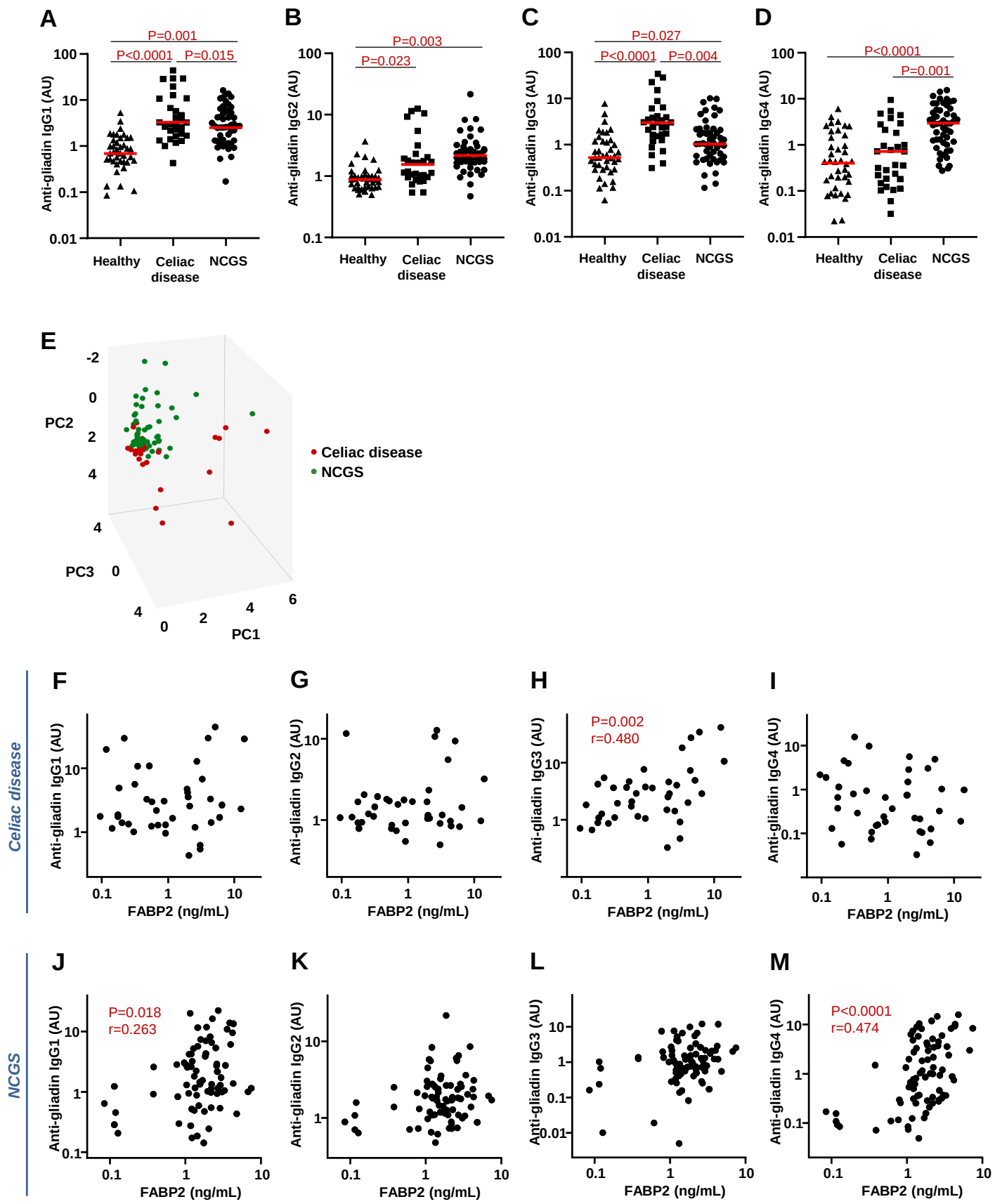

Figure 1 (Revised)

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).

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