# Gliadin Does Not Induce Mucosal Inflammation or Basophil Activation in Patients With Nonceliac Gluten Sensitivity

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**BACKGROUND & AIMS:** **Nonceliac gluten-sensitive (NCGS) patients report intestinal and extra-intestinal symptoms shortly after ingesting gluten; these symptoms disappear on gluten-free diets, although these patients have no serologic markers of celiac disease or intestinal damage. In fact, there is no evidence for mucosal or serologic modifications in those individuals. We investigated immunologic responses of duodenal mucosa samples and peripheral blood basophils, isolated from NCGS patients, after exposure to gliadin.**

**METHODS:** **Participants underwent a complete clinical evaluation to exclude celiac disease while on a gluten-containing diet, a skin prick test to exclude wheat allergy, and upper endoscopy (n = 119) at 2 tertiary medical centers in Italy. Patients were considered to have NCGS based on their symptoms and the current definition of the disorder. Subjects were assigned to the following groups: patients with celiac disease on gluten-free diets (n = 34), untreated patients with celiac disease (n = 35), patients with NCGS (n = 16), or controls (n = 34). Duodenal biopsy samples collected during endoscopy were incubated with gliadin peptides, and levels of inflammatory markers were assessed. Peripheral blood basophils were extracted and incubated with gliadin peptides or a mix of wheat proteins; activation was assessed based on levels of CD203c, CD63, and CD45.**

**RESULTS:** **Duodenal mucosa samples collected from 69 patients with celiac disease showed markers of inflammation after incubation with gliadin. Some, but not all, markers of inflammation were detected weakly in biopsy samples from 3 controls and 3 NCGS patients ($P$ = .00 for all markers). There were no significant increases in the levels of CD63 and CD203c in NCGS patients.**

**CONCLUSIONS:** **Unlike the duodenal mucosa from patients with celiac disease, upon incubation with gliadin, mucosa from patients with NCGS does not express markers of inflammation, and their basophils are not activated by gliadin. The in vitro gliadin challenge therefore should not be used to diagnose NCGS.**

*Keywords:* Allergy; Duodenal Immune Response; Diagnostic; In Vitro Challenge.

Celiac disease (CD) in genetically predisposed individuals is caused by the ingestion of gluten.[1] In CD, specific gluten fractions initiate a T-cell–mediated intestinal mucosal injury[2] ending in mucosal damage, malabsorption of nutrients, and systemic immunologic activation.[3] Diagnosis of CD is based on the evidence of high levels of anti–tissue transglutaminase antibodies (a-tTGs) and/or anti-endomysium antibodies (EMAs) associated with duodenal mucosa damage, regardless of patients' symptoms.[1]

In the past few years, forms of gluten sensitivity other than CD have been gaining the attention of physicians. Some patients have reported the appearance of intestinal (bloating, diarrhea) and extra-intestinal (headache, fatigue/irritability, foggy mind) symptoms shortly after the ingestion of gluten in the absence of any serologic CD marker or intestinal mucosal damage but with a variable presence of antigliadin antibodies (AGA) and the disappearance of such symptoms on a gluten-free diet (GFD). This condition has been defined as nonceliac gluten sensitivity. Two randomized, double-blind, placebo-controlled, re-challenge trials showed that gluten worsened the functional symptoms in nonceliac gluten-sensitive (NCGS) patients in whom CD was excluded.[4,5] Some findings have indicated that in NCGS patients there is a mucosal activation of the innate immunity response without a following adaptative response.[6,7]

Moreover, a recent study[8] showed that the personality and quality of life of NCGS patients were not different from those of

*Abbreviations used in this paper:* AGA, antigliadin antibodies; a-tTG, anti-tissue transglutaminase antibody; CD, celiac disease; EMA, anti-endomysium antibody; FD, free diet; GFD, gluten-free diet; HC, healthy control; ICAM-1, intercellular cell adhesion molecule-1; IBS, irritable bowel syndrome; IgA, immunoglobulin A; NCGS, nonceliac gluten sensitive; TG2, transglutaminase 2.


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CD patients and healthy controls (HCs). NCGS patients were not prone to general somatization; personality and quality of life of NCGS individuals did not differ from those of CD patients or HCs, and NCGS patients claimed more symptoms than CD patients after the gluten challenge.

However, the existence of NCGS as a unique disease condition currently is under debate[9] and requires further investigation.

We recently showed that the gliadin challenge could represent a tool for objective assessment of immunologic modifications in CD, allowing the CD diagnosis in uncommon conditions.[10] In fact, in the presence of gliadin, the duodenal mucosa of celiac patients undergoes several modifications such as crypt hyperplasia, villus atrophy, and T-lymphocyte recruitment in the lamina propria, increasing the number of intraepithelial T lymphocytes[3] that, in part, can be reproduced in vitro and quantified using markers of the early and late inflammatory phase.

Furthermore, studies have shown that incubation of basophils with allergens is followed by a rapid increase in the expression of CD63, a marker of basophil degranulation.[11,12] Typically, NCGS patients report an immediate symptomatic reaction to gluten-containing food; thus we hypothesized that this phenomenon could be explained by basophil degranulation upon contact with wheat protein.

Therefore, the present study evaluates the in vitro wheat protein challenge in 2 experimental settings, duodenal mucosa and peripheral blood basophils, in NCGS patients and compares this with CD patients and HCs.

## Methods

### *Study Design*

The study fulfilled all the items of the Standards for the Reporting of Diagnostic Accuracy Studies statement for diagnostic accuracy (www.stard-statement.org) and was approved by the local ethics committees.

### *Patients*

From January 2010 to July 2011, we consecutively enrolled adult patients (≥18 y) referred to the tertiary centers for food intolerance and CD at the Federico II University of Naples and at the University of Salerno (Italy). Participants signed the informed consent and underwent a dietary interview for evaluation of the gluten content of their diet, upper endoscopy for duodenal biopsies, serum a-tTG levels (enzyme-linked immunosorbent assay; Diamedix, Miami, FL), EMA (EMA IIF; Eurospital, Trieste, Italy) and total IgA evaluation, and assessment of HLA, where appropriate. At the end of the enrollment period, a study cohort was composed of 4 distinct groups of patients: patients without CD (negative serology and histology on a free diet [FD]) reporting irritable bowel syndrome (IBS)-like symptoms after the ingestion of gluten (NCGS patients, N = 16); untreated celiac patients at the moment of diagnosis (eg, on an FD, CD-FD patients, N = 35); treated celiac patients on a gluten-free diet (CD-GFD patients, N = 34); and HCs (N = 34) who underwent laboratory tests and upper endoscopy for reasons other than the suspicion of CD (eg, gastroesophageal reflux symptoms, peptic disease, irritable bowel disease not related to gluten ingestion) to match sex and age (Figure 1). Patients with reported NCGS were

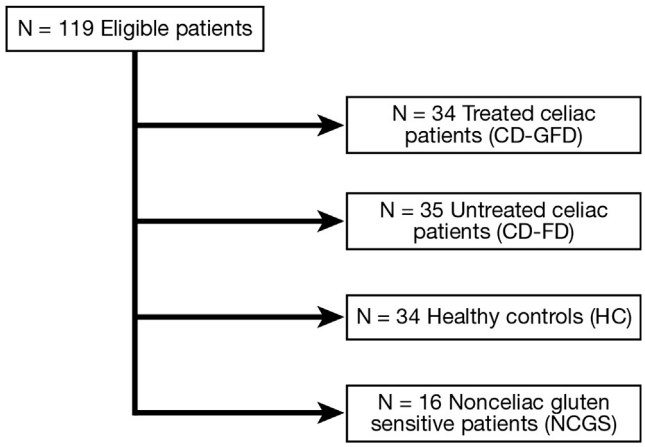


**Figure 1.** Study design. The clinical charts of 119 eligible patients. Patients, according to symptoms, serologic (EMA and a-tTG IgA) and histologic (duodenal biopsies) statuses, were divided into 4 groups.

interviewed carefully by a dietitian expert in CD about their daily diet and about their symptoms before and after gluten withdrawal; they then were classified as NCGS according to the diagnostic criteria suggested by the consensus conference published by Sapone et al.[13] Treated celiac patients had been on a GFD for at least 6 to 12 months after the complete diagnostic work-up including serology and histology. The present study was performed during our previous study assessing the utility of gliadin challenge in cases in which diagnosis of CD was difficult. None of the NCGS patients, however, were included in the previously published report, although their data were collected in the same time period.

NCGS patients, CD patients, and HCs also were tested for wheat allergy using a skin prick test to exclude an IgE-mediated reaction to wheat, as suggested by diagnostic criteria for NCGS.[13] The allergy test was performed using the gliadin fraction C injected subcutaneously in the patient's forearm and was compared with histamine (positive control) and saline solution (negative control) reactions.

### *Gliadin Challenge*

**Duodenal biopsy specimens and tissue culture.** From each patient, duodenal biopsy specimens were collected during the diagnostic upper endoscopy. Two biopsy specimens were oriented on Whatmann paper (Sigma-Aldrich, Milan, Italy) to assess mucosal histology; other specimens were used for gliadin challenge and were placed into an ice-cold tissue-culture medium within 20 minutes. Fragments were placed on a stainless-steel mesh positioned over the central well of an organ-culture dish containing culture medium (37°C), with the epithelium facing upward. The gliadin challenge was performed as previously described, adding a gliadin digest (1 mg/mL) to 4 samples[10] by a biologist blind to the characteristics of the patients. Two fragments were cultured for 3 hours for evaluation of early markers of inflammation, namely: PY99 (anti–phosphotyrosine-monoclonal antibody), HLA-DR (the major histocompatibility complex, class II, DR),[14] and intercellular cell adhesion molecule-1 (ICAM-1).[2,15] Two fragments were cultured for up to 24 hours for evaluation of the delayed markers of inflammation, namely: CD3 (a marker of mature T

**Table 1.** Patient Baseline Status

| | CD-GFD | CD-FD | HCs | NCGS | *P* value |
|---|---|---|---|---|---|
| Patients, n | 34 | 35 | 34 | 16 | |
| Sex, male/female | 4/30 | 5/30 | 4/30 | 3/13 | NS |
| Age, mean ± SD, *y* | 30 ± 11.5 | 30 ± 9.3 | 36 ± 11.5 | 34 ± 9.5 | NS |
| Antibodies status[a] | | | | | .00 |
| EMA positive and a-tTG ND | 11 (32.4%) | 21 (60%) | 0 (0%) | 0 (0%) | |
| EMA ND and a-tTG positive | 0 (0%) | 3 (8.6%) | 2 (5.9%) | 1 (6.3%) | |
| EMA positive and a-tTG positive | 1 (2.9%) | 9 (25.7%) | 0 (0%) | 0 (0%) | |
| EMA negative and a-tTG negative | 21 (61.8%) | 0 (0%) | 31 (91.2%) | 14 (87.5%) | |
| EMA positive and a-tTG negative | 1 (2.9%) | 1 (2.9%) | 0 (0%) | 0 (0%) | |
| EMA negative and a-tTG positive | 0 (0%) | 1 (2.9%) | 1 (2.9%) | 1 (6.3%) | |
| Duodenal histology[a] | | | | | .00 |
| Normal | 1 (2.9%) | 0 (0%) | 9 (26.5%) | 9 (56.2%) | |
| Grade A | 30 (88.2%) | 5 (14.2%) | 25 (73.5%) | 7 (43.8%) | |
| Grade B1 | 2 (5.8%) | 28 (80%) | 0 (0%) | 0 (0%) | |
| Grade B2 | 1 (2.9%) | 2 (5.7%) | 0 (0%) | 0 (0%) | |

NOTE. Patient characteristics are shown in association with diagnosis as number and percentage.
ND, not determined; SD, standard deviation.
[a]$P = .00$.

lymphocytes), CD25 (the inducible interleukin-2 receptor in both lymphoid and myeloid cells), and CD69 (a marker of T-cell activation).[2,15] The remaining 2 fragments served as controls and were cultured similarly for 3 or 24 hours without the addition of gliadin (blank samples). Incubation was stopped by washing, embedding tissues in OTC (Tissue TEK; Milews Laboratories, Elkhart, IN), and snap freezing in cooled isopenthane. Samples were stored in liquid nitrogen and prepared for both histology and immunofluorescence analysis. All samples also underwent a search for transglutaminase 2 IgA (TG2-IgA) deposits before and after 3 and 24 hours.[16]

### *Statistical Analysis*

The parameters investigated were expressed as mean ± SD. The Student *t* test was used to compare data. *P* values less than .05 were considered statistically significant. Analysis of variance was used for comparisons of continuous variables among groups.

## Results

A total of 119 subjects, after written informed consent, agreed to participate in the study. Table 1 reports the descriptive characteristics of our population. Groups were homogeneous for the number of patients (ratio, NCGS:CD; or HC, 1:2), and for age and sex.

### *Serology Status*

Before entry into the study and before starting a GFD, all NCGS patients were EMA negative, but 2 patients showed low-titer a-tTG positivity, in the absence of HLA DQ2 or DQ8. AGA serology was available for 8 NCGS patients, of whom none were AGA IgA positive and 4 were AGA IgG positive with average values not greater than two times the upper limit of normal value (normal value 0–10). As expected, serum a-tTG and/or EMA were positive in all untreated CD (CD-FD) patients and negative in HCs. In treated CD patients, 61.8% were negative for both serum antibodies, and 32.4% were still EMA positive because of the short time on the GFD or because of dietary lapses. Five NCGS patients were DQ2 positive, and 1 patient was DQ8 positive. At the moment of the gliadin challenge, 12 NCGS patients had been on a strict GFD for at least 6 months, and 4 were on a gluten-containing diet. No NCGS patients, CD patients, or HCs tested for gluten allergy showed any kind of skin reaction to gliadin or to saline solution and all reacted against subcutaneous histamine.

### *Duodenal Histology*

Regarding the degree of mucosal damage, it was assessed according to the classification by Corazza and Villanacci[17] by 2 independent pathologists who are experts in CD histopathology. Among NCGS patients, 9 of 16 (56.3%) showed normal mucosal architecture and 7 (43.7%) showed a nonatrophic duodenal modification characterized only by an increased intraepithelial infiltration without any prevalent cellular subpopulation infiltrating the mucosa (eg, eosinophils). None of those patients had villous atrophy, conforming to the diagnostic criteria for NCGS.[13]

### *Gliadin Challenge in Nonceliac Gluten-Sensitive Patients*

As previously illustrated, all biopsy fragments were challenged with gliadin to evaluate immunofluorescence modifications of early and late inflammation markers, and for each marker the difference from baseline was assessed. In our previous study the HLA-DR was found to be the most reliable marker in distinguishing CD patients from other diseases.[10] Figure 2 describes an example of the different immunologic responses of the early marker HLA-DR in CD and NCGS patients. All CD patients, regardless of their dietetic status, showed an increased immunofluorescence intensity, suggestive of mucosal activation, both for early (PY99, epithelial HLA-DR, and ICAM-1) and delayed inflammation markers (crypt HLA-DR, CD3, CD25, and CD69) when stimulated with gliadin. Conversely, only 3 HCs and 3 NCGS patients showed a weak, inconsistent response to the gliadin challenge for some, but not all, inflammation markers, and the mucosa of the majority of them did not seem to be activated by those stimuli (Table 2). Notably, 1 NCGS

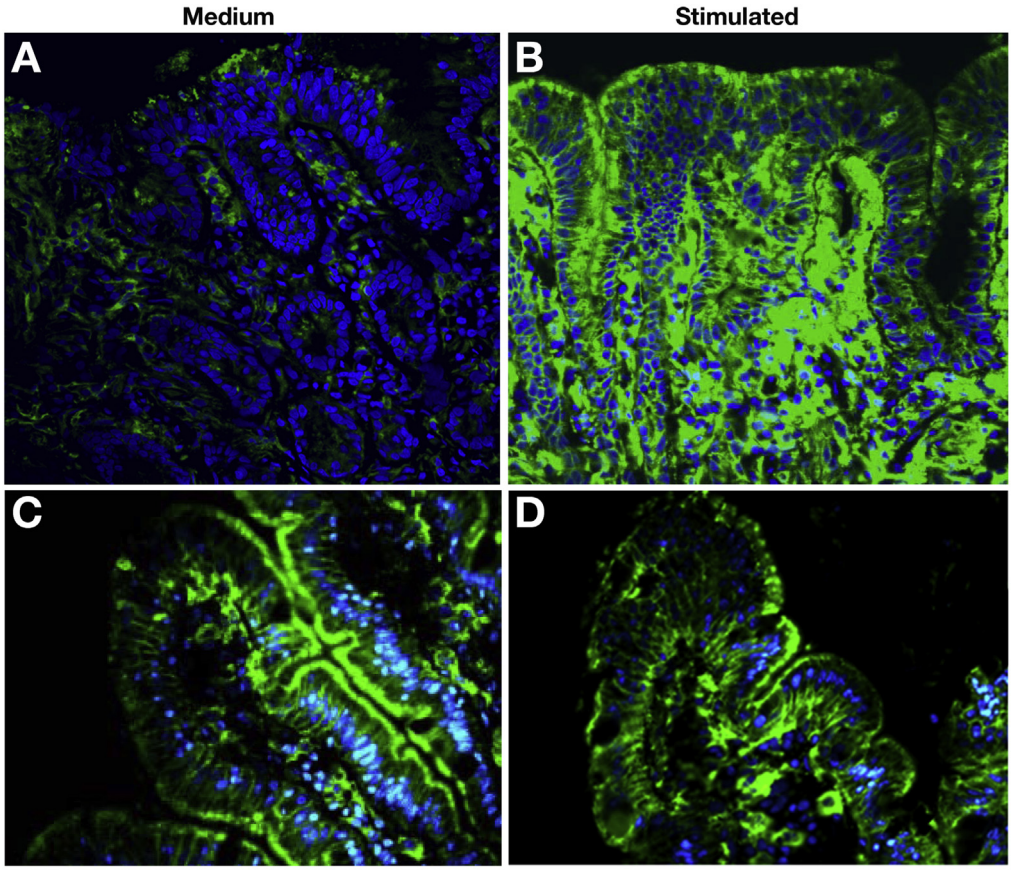


**Figure 2.** Mucosal activation of HLA-DR in CD and NCGS patients. A representative example of the immunofluorescence of mucosal expression of HLA-DR in the presence of gliadin and blank samples. Mucosal activation of HLA-DR marker in (*A*) blank samples and (*B*) after 24-hour challenge with gliadin in a celiac patient and in (*C*) blank samples and (*D*) after gliadin stimuli in an NCGS patient.

patient and 1 control, who showed weak PY-99 and ICAM-1 positivity to gliadin while the other markers tested negative, were found also to have *Helicobacter pylori* infection.

As expected, tissue transglutaminase deposits were present at variable intensity in CD patients but not in NCGS patients and HCs (data not shown).

### *Basophil Activation*

We challenged NCGS patients' basophils with both peptic-tryptic gliadin digest (the same peptides used in the in vitro mucosal challenge) and a mix of wheat proteins (the same as those used in skin prick tests). After stimulation with wheat proteins, the number of activated immunofluorescent basophil markers of cell activation, indicated by levels of CD63 and CD203c expression, were counted and the means were compared. There was no significant difference in the number and characteristics of stimulated basophils when compared with the nonstimulated counterpart and among groups ($P < .004$) (data not shown, examples of cytofluorometry are shown in Figure 3).

**Table 2.** Response to In Vitro Gliadin Challenge Among Groups

| | CD-GFD (n = 34) | CD-FD (n = 35) | HCs (n = 34) | NCGS (n = 16) | *P* value |
|---|---|---|---|---|---|
| Early markers of inflammation | | | | | |
| PY99 | 34 (100%) | 33 (94%) | 1 (2%) | 1 (6.2%) | .00 |
| HLA-DR (epithelial) | 34 (100%) | 34 (97%) | 0 | 0 | .00 |
| ICAM-1 | 34 (100%) | 34 (97%) | 3 (11%) | 3 (18.7%) | .00 |
| Late markers of inflammation | | | | | |
| HLA-DR (crypt) | 34 (100%) | 34 (97%) | 0 | 0 | .00 |
| CD3 | 32 (94%) | 35 (100%) | 3 (8%) | 0 | .00 |
| CD25 | 28 (82%) | 32 (91%) | 2 (5%) | 0 | .00 |
| CD69 | 34 (100%) | 34 (97%) | 3 (11%) | 1 (6.2%) | .00 |

NOTE. Comparison of patients (number and percentage) positive to gliadin challenge for early and late inflammatory markers.

## Discussion

A few years ago, only 1 type of gluten intolerance, CD and its skin form dermatitis herpetiformis, was recognized by the scientific community. Recently, clinical practice indicates that different forms of reaction to wheat may exist. In a clinical study,[5] 276 IBS-like patients who self-reported being intolerant to gluten, negative for both CD and for wheat allergy, underwent a double-blind, placebo-controlled trial, initially with wheat and afterward with cow's milk. The investigators showed that patients who reacted only to wheat (8%) showed characteristics similar to CD patients (eg, DQ2 and DQ8 HLA haplotype, duodenal lymphocytosis, and EMA assay positive in duodenal culture medium). On the other hand, patients who showed multiple food sensitivities (eg, were positive to wheat and cow's milk proteins) had the characteristics of allergic patients (eg, family and personal history of allergy and atopy).

However, even if a clinical form of gluten intolerance other than CD seems to be recognized both by patients and clinicians,

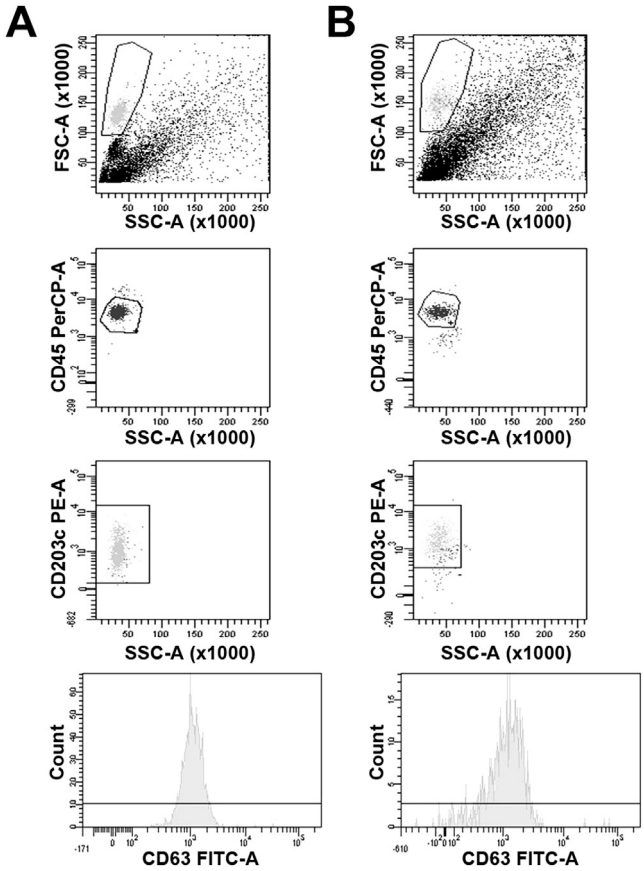


**Figure 3.** Expression of CD63, CD203 C, and CD45 on human stimulated basophils. Representative example of basophil markers' activation in a (*A*) HC and a (*B*) NCGS patient. FITC-A, fluorescein isothiocyanate area; FSC-A, forward scatter area; PE-A, phycoerythrin area; PerCP-A, peridinin chlorophyll protein area; SSC-A, side scatter area.

there is no clear evidence of a mucosal or serologic modification that can be used as stigmata of the disease in those individuals.

In previous studies, the expression of markers of the immune response after in vitro gliadin challenge proved to occur almost exclusively in the mucosa and surnatant of celiac individuals,[10,18,19] hence we applied the same methodology to investigate patients negative for CD but reporting symptoms related to gluten ingestion according to the NCGS definition. The present study shows that in NCGS patients, the gliadin challenge test did not disclose any modification of the expression of mucosal inflammation markers that are found to be increased in CD patients upon gliadin challenge, both in treated and untreated conditions. The NCGS subjects in our series did not show any increase in the tested inflammation markers in the mucosal fragments exposed to a gliadin digest for 3 or 24 hours, in contrast to both treated and untreated CD patients. Therefore, our data indicate that the in vitro gliadin challenge should not be used as a diagnostic tool in the case of NCGS, because in our experiments NCGS patients failed to activate in vitro the same cytokine pathways activated in the case of CD. Recent studies have suggested that NCGS might be the result of the activation of innate immunity not followed by a full response by the adaptive branch of the immune response.[7] However, our experiments showed that PY-99, a marker that recognizes phosphorylated tyrosines, which is an early, common modification of proteins typical of the initiation of innate immunity, does not occur when gliadin is in contact with the intestinal mucosa of NCGS patients.

Furthermore, in contrast with the results of Carroccio et al,[5] we failed to recognize clinical subgroups of NCGS patients with specific diseases because none of our patients showed anemia (data not shown) or skin tests suggesting allergy to gliadin, intraepithelial eosinophilic infiltrate, or personal history of atopy.

Moreover, NCGS peripheral blood basophils stimulated with gluten did not show any sensitivity because the expression of CD63 and CD203c basophils was unchanged in NCGS patients upon allergen challenge. This finding seems to exclude this specific form of cellular sensitivity that was not completely excluded by skin prick tests in our series. The difference with the study by Carroccio et al[5] could be explained by the small number of NCGS patients in our study.

Our study had some limitations: we studied a small number of patients and although 43.7% of the NCGS patients showed an increased number of intraepithelial T lymphocytes, we did not test their mucosa for T-cell receptor $\gamma\delta$ because it was not reported to be increased in NCGS patients.[7] We have explored only some of the possible effects of gliadin challenge in the intestinal mucosa of NCGS patients, limiting our investigation to markers of inflammation that had been studied extensively in CD, and not testing cytokines or other mediators in mucosa or surnatant. The markers tested, however, have the valuable quality of being nonspecific and highly sensitive for detection of early and late phases of intestinal inflammation. Moreover, we did not test all NCGS patients for AGA antibodies because this test was not currently in use for CD diagnosis in adults, although recent studies emphasized the value of AGA antibodies as possible biomarkers of NCGS. Volta et al[20] showed that 45 of 80 NCGS patients under investigation showed IgG AGA positivity, whereas IgA AGA was found in a few patients. If the finding is confirmed in a larger number of NCGS patients, there is the possibility that AGA may represent a biomarker for a subgroup of NCGS patients

In conclusion, in NCGS patients gliadin fractions do not provoke a clear picture of inflammation upon contact with the duodenal mucosa or with peripheral basophils or, if they do, the inflammation is not comparable with that observed in CD patients. Furthermore, our study suggests the possibility that wheat components other than proteins, for example, carbohydrates that already had been associated with the appearance of gastrointestinal symptoms in patients with IBS,[21] also should be investigated for their role in the pathogenesis of NCGS.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at http://dx.doi.org/10.1016/j.cgh.2013.04.022.

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*Conflicts of interest*

**The authors disclose no conflicts.**

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## Supplementary Materials and Methods

### *Protein Extraction and Digestion*

Ground kernels (100 g) of bread wheat (*Triticum aestivum*) cultivar Bolero were mixed with 400 mL (wt/vol) of distilled water, stirred at 4°C for 1 hour, and then centrifuged at 5000 × *g* for 10 minutes at 4°C. The supernatant containing albumins (fraction A) was transferred to a fresh tube, whereas the pellet was suspended in 300 mL of 0.5 mol/L NaCl, stirred at 4°C for 1 hour, and then centrifuged at 5000 × *g* for 10 minutes at 4°C. The supernatant containing globulins (fraction B) was transferred to a fresh tube, whereas the pellet was suspended in 300 mL of 70% ethanol, extracted for 1 hour at room temperature under stirring, and centrifuged at 5000 × *g* for 10 minutes at 20°C. The supernatant containing gliadins (plus a certain amount of low-molecular-weight glutenin polymers) (fraction C) was transferred to a fresh tube, whereas the pellet was suspended in 300 mL of 0.5 mol/L acetic acid, extracted for 1 hour at room temperature under stirring, and centrifuged at 5000 × *g* for 10 minutes at 20°C. The supernatant containing high-molecular-weight glutenin polymers (fraction D) was transferred to a fresh tube. The albumin, globulin, gliadin, and glutenin proteins (A–D fractions) in the fresh tubes were precipitated overnight with 2 volumes of acetone at −20°C, frozen, and freeze-dried. The 4 protein fractions were submitted to peptic-tryptic sequential digestion as described by De Ritis et al.[1] At the end of the procedure, the peptic-tryptic digests were heated for 30 minutes at 100°C, lyophilized, and stored at −20°C.

### *Basophils Activation Assay*

**Immunophenotyping of basophils by flow cytometry.** Basophils were obtained from EDTA containing peripheral blood. After isolation, cells ($1 \times 10^6$) were stimulated with media alone, the peptic-tryptic digest of fractions A (albumin) and B (globulin) combined, and the peptic-tryptic digest of gliadin fraction C. Mixed proteins and gliadins (same mixture as used in skin prick tests) were diluted in 70% ethanol (stock solution: 1 mg/mL), for 0, 5, 10, and 15 minutes at 25, 50, and 80 μg/mL, and with peptide digest (peptic-tryptic digest, 1 mg/mL). We analyzed the expression of hematopoietic membrane antigens anti-CD203c phycoerythrin (clone NP4D6; BioLegend, San Diego, CA), anti-CD63 fluorescein isothiocyanate (clone MEM-259; BioLegend), and anti-CD45 peridinin chlorophyll protein complex (clone H130; BioLegend) on the surface of basophils by flow cytometry. For all antibody staining experiments, cells were incubated at 4°C for 20 minutes with the appropriate amount of monoclonal antibodies, lysed with ammonium chloride ($NH_4Cl$), washed twice with phosphate-buffered saline, and, finally, analyzed with an unmodified FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA), that was set up according to published guidelines. For each sample, the respective control was prepared to determine the level of cellular autofluorescence background without antibody staining. Samples were acquired immediately after staining using the FACSCanto II instrument, and at least 30,000 events were recorded for each monoclonal combination. Levels of CD antigen expression were displayed as median fluorescence intensity. FACS-DiVa software (Becton-Dickinson) was used for cytometric analysis. Isolation and enrichment of basophils was obtained using the Human Basophil Enrichment Kit, performed according to the manufacturer's instructions (EasySep Human Basophil Enrichment Kit; Stem, STEM CELL Technologies Inc, Vancouver, Canada).

### *Immunofluorescence*

For analyses of antigen expression and tissue distribution by indirect immunofluorescence, 4-μm cryosections were incubated separately in the presence of the following antibodies: PY99 (1:80, mouse IgG2b; Santa Cruz Biotechnology, Santa Cruz, CA); HLA-DR (1:10, Becton-Dickinson); ICAM-1 (1:400; Ylem, Rome, Italy); CD3 (1:100; Dako, Glostrup, Denmark); CD25 (1:30; rabbit polyclonal; Dako); and CD69 (1:80; Dako). Antigen expression and distribution was visualized using a donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (Becton-Dickinson) for 60 minutes at room temperature. Two color immunofluorescences with anti-CD25 and anti-CD3 antibodies were performed as previously described.[2] Isotype control antibodies (IgG1 or IgG2), isotype-matched nonimmune IgGs, or isotype-matched antibodies against inappropriate blood group antigens were used as controls of specificity. Data were analyzed under fluorescence examination using a Leica DM2005 microscope (Leica Microsystems, Wetzlar, Germany). HLA-DR expression was graded from absent to very strong (scale, 0–3); ICAM-1–, CD3/CD25-, and CD69-positive cells were counted per $mm^2$ of mucosa. The number of epithelial cells stained with PY99 was counted per 100 epithelial cells (0, less than 25%; 1, 25%–49%; 2, 50%–74%; 3, 75% and greater). The TG2 deposit search was performed by double labeling for human IgA (green) and for TG2 (red) with the use of monoclonal mouse antibodies against TG2 (CUB7402; NeoMarkers, Fremont, CA). The sections were washed in phosphate-buffered saline and incubated with a mixture of fluorescein isothiocyanate–labeled rabbit antibody against human IgA (1:100; Dako) to detect (in green) IgA deposits and R-phycoerythrin–labeled rabbit antimouse antibody (1:40; Dako) to detect (in red) TG2, for 30 minutes in the dark. Finally, the sections were washed several times in phosphate-buffered saline and mounted by glycerol/phosphate-buffered saline (1:10). The test was considered positive when there was an increase, compared with blank samples, of 1 or more arbitrary units or in counts of positive cells for all markers with the exception of TG2-IgA deposits. IgA deposits and TG2 were considered positive in the case of subepithelial IgA deposition below the basement membrane along the villous and crypt epithelium and around mucosal vessels; faint immunostaining was considered negative.

Assessment and grading of immunofluorescence intensity was performed as follows: for each marker, mucosal activation was considered positive when gliadin-stimulated markers showed a change of at least 1 point higher than the baseline (eg, non–gliadin-stimulated) counterpart. Then the number of reacting patients (eg, patients who effectively showed a difference in mucosal immunofluorescence intensity between basal and 3- or 24-hour gliadin-challenged mucosa) were compared among the 4 groups.[2]

### Supplementary References

1. Auricchio S, De Ritis G, De Vincenzi M, et al. Effects of gliadin-derived peptides from bread and durum wheats on small intestine cultures from rat fetus and coeliac children. Pediatr Res 1982; 16:1004–1010.
2. Tortora R, Russo I, De Palma GD, et al. In vitro gliadin challenge: diagnostic accuracy and utility for the difficult diagnosis of celiac disease. Am J Gastroenterol 2012;107:111–117.