More fuel to the fire: Some patients with non-celiac gluten sensitivity exhibit adaptive immunological responses in duodenal mucosa

Antonia Isabel Castillo-Rodal
Universidad Nacional Autonoma de Mexico Escuela Nacional Preparatoria Plantel 9 Pedro de Alba

Janette Furuzawa-Carballeda
Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Mario Pelaez-Luna
Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Jose Castro-Gomez
Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Yolanda Lopez-Vidal
Universidad Nacional Autonoma de Mexico

Luis Federico Uscanga (✉ luis.uscangad@gmail.com )
Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

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Abstract

Background

In contrast to the well characterized Celiac Disease (CD), the clinical scenarios encompassed in non-celiac gluten sensitivity (NCGS) might be related to different antigens that trigger distinct immune-inflammatory reactions. Although an increased number of intestinal intraepithelial lymphocytes is observed at the inception of both diseases, subsequent immunopathogenic pathways seems to be different.

Aims

To compare the immunological profile in the duodenal mucosa of patients with CD, self-reported gluten intolerant subjects and gluten tolerant patients with functional dyspepsia (GT-FD).

Methods

In a blind, cross-sectional study, duodenal biopsies from 15 consecutive untreated patients with active CD, 9 NCGS individuals and 10 GT-FD subjects were studied by flow-cytometry and immunohistochemistry. We determined the presence of pro-inflammatory cytokine expressing monocytes and monocyte-derived dendritic cells involved in innate immune activation, cytokine-driven polarization and maintenance of Th1 and Th17/Th22, and anti-inflammatory/profibrogenic cytokines.

Results

CD patients presented a higher percentage of cells expressing all tested cytokines in lamina propria and epithelium than GT-FD group. Cytokines that induce and maintain Th1 and Th17 polarization were higher in CD compared to NCGS and GT-FD cases; and higher in NCGS compared to GT-FD. Similar differences in the expression of IL-4 and TGF-β1 were detected, while IL-10-expressing cells were lower in NCGS patients compared to CD and GT-FD subjects.

Conclusions

NCGS patients exhibit components of both, innate and adaptive immune mechanisms but to a lesser extent compared to CD. The clinical characteristics and HLA status of our NCGS group resemble that described in subjects with irritable bowel syndrome sensible to gluten and probably represents a distinct phenotype of this syndrome.

Introduction
Celiac disease (CD) and non-celiac gluten sensitivity (NCGS) are gluten related disorders (GRD) that share clinical characteristics but have marked serological and histological differences.\textsuperscript{1-3} While autoantibodies and duodenal villus atrophy (VA) must be present in order to diagnose CD, they have to be absent in order to establish a presumptive diagnosis of NCGS.

Due to the lack of specific biomarkers the diagnosis of NCGS is largely based upon clinical characteristics with symptoms improving during a gluten-free diet and symptomatic relapse while consuming a gluten-containing diet, along with normal duodenal biopsies and negative CD serology. In these cases, wheat allergy should be also discarded.\textsuperscript{4, 5}

CD is a well-characterized disease with specific histological and serological features and convincing immunopathology mechanisms triggered by the ingestion of gluten and related proteins. In genetically predisposed individuals, gluten ingestion results in mucosal inflammation and VA mediated by a series of processes orchestrated by CD4+ T helper 1 (Th1) and Th17 cells.\textsuperscript{6-10}

In contrast, NCGS is a poorly characterized disorder in which the role of gluten as the main antigen and the pathophysiologic mechanisms involved in tissue damage and symptoms development are debatable. Actual evidence suggests a paramount role of the innate immune response, although some authors have found that the adaptive immune response may be involved.\textsuperscript{6,11-15}

The main objective of this work is to compare the immune response in duodenal mucosa of celiac patients with that of self-described gluten intolerant subjects. Although an increased number of intestinal intraepithelial lymphocytes is observed at the inception of these entities, subsequent immunopathogenic pathways seems to be different. Therefore, we compared the immunologic profile of the duodenal mucosa of subjects with CD, NCGS and a control group of gluten tolerant subjects with functional dyspepsia (GT-FD) focusing in the pro-inflammatory cytokine-expressing monocyte and monocyte-derived dendritic cells involved in innate immune activation, cytokine-driven polarization and maintenance of Th1 polarization and Th17/Th22, and anti-inflammatory/profibrogenic cytokines.

**Methods**

**Patients and controls**

In this blind and cross-sectional study, we included 15 consecutive untreated patients with active CD and 9 individuals with NCGS who attended to the Department of Gastroenterology outpatient clinic at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, a tertiary care medical facility in Mexico City.

CD was diagnosed when patients met the following criteria: 1) Compatible clinical data: chronic diarrhea, weight loss, bloating, abdominal disconfort, fatigue or, nutrient deficiencies, 2) positive serum autoantibodies: *anti-endomysium* (EmA IgA), *anti-transglutaminase* (anti-tTg IgA) and *anti-deamidated gliadin peptide antibodies* (AGA-DGP IgA and IgG) and, 3) VA according to Marsh-Oberhuber criteria.\textsuperscript{16}
Diagnosis of NCGS was considered when patients presented with: 1) intestinal and extra intestinal symptoms associated with the ingestion of gluten-containing food, 2) a clear clinical response while they followed a gluten-free diet (GFD), 3) relapse of symptoms with the ingestion of gluten-containing foods, 4) negative CD (EmA IgA, anti-tTG IgA, AGA-DGP) and wheat allergy (IgE serological test) serological markers and, 5) normal duodenal biopsies.

All patients were evaluated by a qualified nutritionist with expertise in CD. Symptoms severity (abdominal discomfort or pain, bloating, diarrhea, and constipation) was assessed using a visual analog scale (VAS: 0-10) at baseline, while on an unrestricted diet, 6 weeks after they followed a GFD and after concluding a 6 week challenge based on a diet adjusted to contain 10 grams of gluten par day. Diet compliance was evaluated at biweekly out-patient visits during the gluten-challenge. All serological tests (EmA IgA, anti-tTG IgA, AGA-DGP) were performed at baseline visit and at conclusion of the 6 weeks gluten challenge. The presence of headache, tingling or numbness on feet or hands, fatigue, musculoskeletal pain, brain fog, rash and oral ulcers were considered extra-intestinal symptoms and were specifically evaluated.

We considered a good clinical response to the GFD when there was a decrease in the intensity of symptoms of at least 50% compared to the basal VAS. We did not perform a double-blind gluten/placebo-controlled trial challenge in any case.

We excluded patients with other gastrointestinal diseases, history of gastrointestinal surgery, active or previous infectious diseases, clotting disorders, renal insufficiency, pregnancy or breast feeding, active use of antimicrobial, probiotics, immunosuppressive drugs, non-steroidal anti-inflammatory drugs or corticosteroids.

As a control group we included 10 subjects that fulfilled ROME III criteria for functional dyspepsia (FD) with negative CD serology and who had undergone an upper endoscopy with normal duodenal histology (GT-FD).

**Biopsy Sampling**

During upper endoscopy four tissue samples from the second portion of the duodenum were obtained, two of them were placed immediately in ice-chilled Hank buffer solution (HBSS) /5% fetal bovine serum (SFB, GIBCO). The others were fixed in 10% formaldehyde and subsequently embedded in paraffin wax and cut into 4 μm thick sections.

**Intraepithelial lymphocytes (IELs) Isolation from Duodenal Tissue**

Duodenal mucosa samples (epithelium and lamina propria) were cut with a scalpel blade and incubated in phosphate buffer 1x (PBS) / ethylenediamine tetra acetic acid (EDTA) 2mM at 34°C for 30 min while being agitated. After that, samples were treated with Collagenase IV (Sigma) at 60 U/ml for 1 h at 34°C while being agitated. The cell suspension was then passed through a 40μm cell strainer (Cell Strainer BD Falcon), washed with 2 ml of PBS, and centrifuged at 800 g for 10 min at 25°C. The resulting pellet was homogenized in 1 mL of PBS and incubated with 1 μL of Brefeldin A (BD Golgi Plug) for 1 h at 37°C with
5% CO2. Live-dead assay and cellular count was realized on cellular samples (>90%) on Neubauer chamber (trypan blue).

**Immunohistochemistry**

Tissues placed on positively charged slides were incubated with mouse monoclonal anti-human IL-1β, IL-6, IL-8, IL-10, IL-15, IL-22, IL-23, IFN-γ, TNF-α, and with rabbit polyclonal anti-human IL-2, IL-12p40, IL-17A, IL-21, or TGF-β1 antibody (Abcam, Cambridge, MA, USA) or anti-human IL-4 antibody (Bio Legend Inc., San Diego, CA, USA) at 10 µg/mL during 30 min. Binding was detected with Universal Dako labelled streptavidin biotin reagent+peroxidase for primary antibodies from rabbit, mouse and goat (Dako, Glostrup, Denmark). Spleen and ganglion samples were used as a positive control. Negative controls were carried out with normal human serum (1:100) and with the IHC universal negative control reagent (Enzo Life Sciences, Inc., Farmingdale, NY, USA), while phosphate buffer saline-egg albumin (SIGMA-Aldrich) was use in the reactive blank. Controls excluded nonspecific staining or endogenous enzymatic activities. We examined three different sections of each biopsy. Cytokine-expressing cells were reported as the percentage of positive cells in three fields (X320) taken from the epithelium and lamina propria. Results are expressed as the median, mean and 5th/95th percentiles.

**Peripheral Blood Mononuclear Cells (PBMCs) Isolation.**

We collected a sample of venous blood to isolate PBMCs by gradient centrifugation on Ficoll-Paque (Merck-Millipore). The bottom was resuspended in 1 mL of PBS 1x/Brefeldin A (BD GolgiPlug) and incubated at 37°C in 5% CO₂ during 1h. Live-dead assay (trypan blue) and cellular count was realized on cellular samples (>90%).

**Flow Cytometry.**

1X10⁵ PBMCs or mLs were labeled with 5 µL of antihuman CD4-FITC-labeled, monoclonal antibody (BioLegend San Diego, CA). Cells were permeabilized with 200 µL of cytofix/cytoperm solution (BD Biosciences). Intracellular staining was performed with an anti-human Foxp3-PE-, IFN-γ-APC-Cy7-, IL-17A-PE-Cy7- (BioLegend), T-bet-PerCP-Cy5.5- (BD Pharmingen, San Jose, CA), and ROR-γt-APC-labeled (R&D Systems, Minneapolis, MN) mouse monoclonal antibodies. From the electronic bi-parametric gate of the singlets and living cells, we performed an analysis in the CD4+ lymphocytes population to identify CD4+/Foxp3+ cells, CD4+/T-bet cells, CD4+/INF-γ cells, CD4+/ROR-γt+ cells, CD4+/IL-17A cells. Results are expressed as the relative percentage of CD4+/IL-17A−, CD4+/INF-γ−, CD4+/Foxp3−, CD4+/T-bet−, and CD4+/ROR-γt−-expressing cells in each gate. For an auto fluorescence control, we ran an unstained and permeabilized cell sample. An AbC anti-mouse bead kit (Invitrogen, UK) was used to adjust instrument settings, to set fluorescence compensation, and to check instrument sensitivity. Fluorescence minus one (FMO) controls were stained in parallel. Samples were analyzed with an Attune Acoustic Focusing Cytometer Blue/Red (Life Technologies). We recorded more than 10,000 events for each sample, and they were analyzed with Attune® Cytometric Software v2.1.
Ethical Considerations. This work was performed according to the principles expressed in the Declaration of Helsinki. Our Institutional research and ethics committees approved the protocol (GAS-1298-14/15-1; August 11, 2014). Each patient signed a written informed consent.

Statistical analysis

Data were analyzed using GraphPad Prism for Windows (version 6.01 GraphPad software Inc. USA) and Kruskall Wallis non-parametric test. Immunohistochemical data are expressed as the median, mean and 5th/95th percentiles. We performed one-way ANOVA on ranks by Holm-Sidak method and Dunn's test for all pairwise multiple comparison procedures. A p value <0.05 was considered statistically significant.

Results

Demographic and clinical characteristics are summarized in Table 1 and 2. Diarrhea, abdominal pain and bloating were the most frequent symptoms in both, CD and NCGS patients. All NCGS patients’ symptoms improved during the GFD but while on the gluten challenge, they presented with bloating and abdominal pain. One of them complained from mouth itching. Nonetheless all completed the challenge.

In 2 patients, the levels of AGA-DGP IgA (NCGS) and AGA-DGP IgG (control group) were above the upper limit of normal (ULN). None of them showed other alterations neither in serology nor in histopathology.

Percentage of Peripheral CD4+ T cell subpopulations in PBMCs

No differences were observed in the number of CD4+ T cells (Figure 1A), CD4+/Foxp3+ (Figure 1C), CD4+/T-bet+ (Figure 1E), CD4+/IFN-γ+ (Figure 1G), CD4+/ROR-γt+ neither CD4+/IL-17A+ cells (Figure 1K) amongst the groups.

Percentage of mucosal subpopulations in duodenal tissue

The percentage of CD4+ mls was higher in CD patients compared to the control group (P=0.003, Figure 1B). No differences were found between CD patients and NCGS nor between NCGS and the control group (GT-FD).

CD patients had a significantly higher CD4/FoxP3 percentage in duodenum compared to the control group. (P=0.039, Figure 1D). The NCGS CD4/FoxP3 percentage was similar to both, CD and control group.

T-bet and ROR-γt were higher in CD patients versus control group (P=0.036, Figure 1F and P=0.03, Figure 1J, respectively). No differences were observed between CD patients and NCGS. Neither there was any difference when comparing NCGS to the control group. Moreover, the percentage of IFN-γ− and IL-17A−expressing CD4 mucosal T cells in the CD group, although higher than NCGS, was not statistically significant amongst the three groups (Figure 1H and 1L).
Pro-inflammatory cytokines in duodenal tissue

The percentage of IL-1β- and TNF-α-expressing cells in tissue of CD and NCGS patients was significantly higher compared to the control group. It is noteworthy, that tissue of NCGS patients had statistically significant lower levels of IL-1β- and TNF-α-expressing cells compared with CD patients (Figure 2A,B).

The number of IL-6- and IL-8-producing cells was significantly higher in CD patients compared with control group and NCGS patients. No differences in the number of IL-6+ or IL-8+ cells were found in the NCGS group compared to the control group (Figure 2C,D).

Cytokines involved in the differentiation and maintenance of Th1 in duodenal tissue

The percentage of IL-2+ and IFN-γ+ cells of CD patients was higher compared to NCGS and control group. No statistically significant differences in the number of IL-2+ or IFN-γ+ cells were determined in the NCGS group compared to the control group (Figure 3A,D).

The IL-12 and IL-15 cell percentage of CD patients was conspicuously higher when compared to the control group and NCGS. Interestingly, the number of IL-12- and IL-15-expressing cells was significant higher in NCGS compared to control (Figure 3B,C).

Cytokines involved in the differentiation and maintenance of Th17/Th22 in duodenal tissue

The percentage of IL-17A+, IL-21+, IL-22+ and IL-23+ cells of CD patients was higher versus control group, and NCGS patients (Figure 4A-D). It is noteworthy, that tissue of NCGS group had statistically significant lower levels of IL-17A-, IL-21-, IL-22- and IL-23-α-expressing cells compared with CD patients (Figure 4A-D).

Anti-inflammatory/Profibrogenic cytokine expression in duodenal

No differences were observed in IL-4 cell percentage when compared CD patients or NCGS versus control group (Figure 5A).

TGF-β1- and IL-10-expressing cells from CD or NCGS patients were higher versus control group (Figure 6B,C). However, no statistically significant difference was found between CD and NCGS patients.

Discussion

CD is a well-characterized disease with specific histological and serological features and established immuno-pathological mechanisms triggered by the ingestion of gluten and related proteins, affecting genetically predisposed individuals.7, 10 In contrast, NCGS is a disorder seeking its own identity. It is a condition that encompasses different clinical scenarios including subjects with irritable bowel syndrome (IBS) and patients with food intolerances. NCGS abdominal and extra-intestinal troubles trigger by foods
containing gluten and symptoms typically improve with a GFD. The ambiguity about its existence is based on the absence of specific biological markers and histological characteristics. Its diagnosis has been based on information from complex and heterogeneous double-blind placebo-controlled challenges that used different vehicles and doses of gluten.

In an attempt to standardize NCGS diagnosis, a group of experts met in Salerno and based on the results of a double-blind placebo-controlled challenge using 8 grams of gluten administered over 2 periods of one week separated by one-week wash-up term proposed an expert’s diagnostic criteria.

Since the Salerno criteria is difficult to fulfill in the clinical setting, once CD and wheat allergy have been reasonably ruled out, the presumptive diagnosis of NCGS can be considered and is based merely on the clinical responses to a diet with and without gluten.

Despite these diagnostic caveats, we are confident that our self-considered gluten intolerant patients and, here classified as NCGS, have wheat intolerance considering that all of them improved while on a GFD and relapsed when they followed a gluten-containing diet challenge. CD and wheat allergy were reasonable excluded through serological test and histological features. Since we did not perform a double-blind gluten/placebo-controlled challenge in any case, the role of other antigens besides the gluten present in wheat cannot be excluded.

While on an unrestricted diet, flow cytometry and immunohistochemical assessment showed that CD4+ cell subpopulations were quite different among groups.

As expected, in flow cytometry, CD patients exhibited a wealth range of innate and adaptive immune responses when compared to the control group. The percentage of inflammatory and regulatory cells CD4+ T, CD4+/Foxp3+, CD4+/T-bet+, CD4+/ROR-γt+ were higher in CD than in the control group and NCGS patients. Importantly, the percentage of inflammatory and regulatory CD4+ cells was higher in the NCGS patients compared to the control group but lower than that observed in CD subjects. Although this finding did not reach statistically significant difference, it coincides with prior reports, suggesting that an inflammatory process is present in these self-reported gluten intolerant subjects.

Immunohistochemical analysis showed the most noticeable changes. The percentage of pro-inflammatory cytokine-expressing cells in the duodenal mucosa was higher in patients than in controls. The inflammatory response was conspicuously higher in CD subjects. Pro-inflammatory cytokine-expressing cells were also evident in NCGS patients, except for IL-6- and IL-8-expressing cells.

Present evidence suggests that innate immune response plays a central key role in the pathophysiology of NCGS. Different authors have reported an increased expression of toll-like receptor 2 (TLR-2) and 4 (TLR-4), claudin 4 (CLD-4), IL-8 and TNF-α in subjects with self-reported gluten intolerance. According to this concept, IL-1- and TNF-α-expressing cells were higher in NCGS patients compared to the control group. All these findings support the widely demonstrated participation of innate immunity in both, CD and NCGS.
Th cell polarization from naïve precursor is a tightly controlled process, where IL-12 and IL-15 play a central role as factors involved in the differentiation of Th1 response.\textsuperscript{25, 26} The secreted IL-2 by activated antigen-specific CD4+ and CD8´T cells is consumed at the same and distant sites by cells that express the IL-2Rα (e.g. effector T cells, NK cells and Treg). IL-2 acts via STAT5 and influences the differentiation of Th1, Th2 and Th17 cell subsets, also it is important to maintain the transcriptional program for the Treg function.\textsuperscript{27}

In addition, IL-2 probably stimulates the differentiation of other cell groups while IL-12 does it for Th1 and, IL-22 and IL-23 for Th17. In contrast, IL-15 not only promotes the increase of IEL´s in CD but also supports Th1 and Th17 responses.\textsuperscript{25, 28-30} Meeting these concepts, we observed that the percentage of cytokine expressing-cells that induce and maintain Th1 and Th17 polarization in the mucosa of CD patients was higher compared to the other groups. Interestingly, IL-2- and IL-12-expressing cells were also increased in NCGS group compared to control group, suggesting some participation of adaptive immunity components in these self-reported wheat intolerant subjects.

Recent evidence supports the participation of adaptive immune mechanisms in NCGS. Mucosal based studies have found high levels of IFN-γ in duodenum and rectum of NCGS patients challenged with gluten.\textsuperscript{(31,32)} Moreover, it has been shown that these patients are able to produce specific antibodies to native gliadins and, at least 50% of them compared to 30% of the general population, express HLA-DQ2/DQ8, haplotypes the risk for CD.\textsuperscript{(33)}

It should be noted that we found haplotypes of risk for CD in 6 of 8 patients with NCGS while all CD subjects had HLA DQ2/DQ8 genes.

TGB-β1-expressing cells were notably higher in both, CD and NCGS patients. It is very likely that this finding is an attempt to control the inflammatory process elicited by peptides from prolamines in the case of CD or other antigens recognize in NCGS patients\textsuperscript{(34)}. Interestingly IL-10-expressing cells were not increased neither in CD nor in NCGS. In fact, they were lower in NCGS than in the other groups.

Our findings in CD patients are quite similar to those widely reported in the literature but some differences can be noted in our NCGS patients. At the present time the term NCGS represent an umbrella covering a wide range of unspecific non-celiac clinical scenarios. Our patients with NCGS were diagnosed on clinical bases excluding wheat allergy and CD, however, we did not perform a double-blind gluten-placebo challenge in any case and the participation of other antigens, besides gluten, could not be excluded. It could be argued that in fact they could represent different forms of CD, perhaps seronegative CD, mainly because the majority present with HLA DQ2/DQ8 genotype, however all of them had a normal duodenal mucosa while on a gluten-containing diet.\textsuperscript{(35)}

Due to the mentioned NCGS diagnostic flaws, these patients represent a true challenge in clinical practice. In the absence of a double-blind gluten/placebo- controlled challenge suggested by the Salerno expert consensus, it seems reasonable to consider them as patients with wheat intolerance as it has already been suggested\textsuperscript{(17,18)}. At the present time it seems clear that other wheat components like
fermentable short-chain carbohydrates (FODMAP) or amylase-trypsin inhibitors (ATI) play a role in eliciting symptoms and inflammatory response mainly mediated by innate immunity (15,34). In this work we found that our patients with NCGS exhibited components of both innate and adaptive immunity. We believe that these findings provide one more piece to the complicated puzzle of wheat sensitivity disorders.

**Conclusions**

Some self-reported gluten intolerant subjects have components of both, innate and adaptive immunity response in the duodenal mucosa. The clinical characteristics and HLA genotype of our patients resemble to that described in subjects with IBS-D sensible to wheat and it probable represent a distinct phenotype of IBS.

**Abbreviations**

Celiac disease

NCGS. Non-celiac gluten sensitivity

GT-FD. Gluten tolerant patients with functional dyspepsia

IL-4. Interleukin 4

TGF-β1. Tumor growth factor β1

IL 10. Interleukin 10

HLA. Human leukocyte antigen

GRD. Gluten related disorders

Villous atrophy

Th1. T helper 1 lymphocytes

Th17. T helper 17 lymphocytes

Th22. T helper 22 lymphocytes

EmA IgA. Anti-endomysium antibodies

Anti-tTg IgA. Anti-transglutaminase antibodies

AGA-DGP IgA and IgG. Anti-deamidated gliadin peptide antibodies
GFD. Gluten free diet.

VAS. Visual analog scale

Functional dyspepsia

HBSS. Hank buffer solution

SFB. Fetal bovine serum

IEL. Intraepithelial lymphocytes

PBS. Phosphate buffer

EDTA. Ethylenediamine tetra acetic acid

IL-1β. Interleukin 1 β

IL-6. Interleukin 6

IL-8. Interleukin 8

IL-15. Interleukin 15

IL-22. Interleukin 22

IL-23. Interleukin 23

IFN-γ. Interferon γ

TNF-α. Tumor necrosis factor α

IL-2. Interleukin 2

IL-12p40. Interleukin 12p40

IL-17A. Interleukin 17A

IL-21. Interleukin 21

PBMC. Peripheral blood mononuclear cells

ULN. Upper limit of normal

IBS. Irritable bowel syndrome

TLR-2. Toll-like receptor 2
Declarations

Ethics approval and consent to participate

The protocol was approved by Research and Ethics Committees of Instituto Nacional de la Nutrición Salvador Zubirán (GAS-1298-14/15-1; August 11, 2014). Each patient signed a written informed consent to participate.

Consent for publication

“Not applicable”

Availability of data and materials

The datasets during or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

None to report.

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Author’s contributions

A.I.C.-R., and L.U. conceived and designed experiments. J.F.-C. Performed experiments. J.C. recruited the patients and controls. A.I.C.-R., L.U., J.F.-C., M.P, Y.L.-V, and J.C.-G. Analyzed data. A.I.C.-R., M.P, L.U., and J.F.-C. Wrote and edited the manuscript.

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Author details

1 Department of Microbiology and Parasitology. Facultad de Medicina. Universidad Nacional Autónoma de México. Alcaldía de Coyoacán. Ciudad de México. México.

2 Department of Immunology and Rheumatology. Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga 15. 14000. Alcaldía de Tlalpan. Ciudad de México. México;

3 Department of Gastroenterology. Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga 15. 14000. Alcaldía de Tlalpan. Ciudad de México. México

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Tables

STROBE Statement—Checklist of items that should be included in reports of cross-sectional studies
More fuel to the fire: Some patients with non-celiac gluten sensitivity exhibit adaptive immunological responses in duodenal mucosa

| Title and abstract | 1 |
|-------------------|--|
| Page 2. Paragraph 3 |
| Page 2. Paragraph 4 |

(a) Indicate the study's design with a commonly used term in the title or the abstract

(b) Provide in the abstract an informative and balanced summary of what was done and what was found

Introduction

Background/rationale

Page 3. Paragraph 4

Explain the scientific background and rationale for the investigation being reported

Objectives

Page 4. Paragraph 5

State specific objectives, including any prespecified hypotheses

Methods

Study design

Page 5. Paragraph 1

Present key elements of study design early in the paper

Setting

Page 5. Paragraph 1

Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection

Participants

Page 5. Paragraph 2,3
Page 6. Paragraph 3

(a) Give the eligibility criteria, and the sources and methods of selection of participants

Variables

"N/A"

Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable

Data sources/ measurement

"N/A"

For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of
| Assessment Methods | Page | Description |
|--------------------|------|-------------|
| Bias “N/A”         | 9    | Describe any efforts to address potential sources of bias |
| Study size “N/A”   | 10   | Explain how the study size was arrived at |
| Quantitative variables | 11  | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why |
| Statistical methods | 12   | (a) Describe all statistical methods, including those used to control for confounding<br>(b) Describe any methods used to examine subgroups and interactions<br>(c) Explain how missing data were addressed<br>(d) If applicable, describe analytical methods taking account of sampling strategy<br>(e) Describe any sensitivity analyses |

**Results**

| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed<br>(b) Give reasons for non-participation at each stage<br>(c) Consider use of a flow diagram |
|--------------|-----|--------------------------------------------------|
| Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders<br>(b) Indicate number of participants with missing data |
| Section                  | Page | Paragraph | Page | Page |
|--------------------------|------|-----------|------|------|
| Outcome data             |      |           |      |      |
| “N/A”                    | 15*  |           |      |      |
|                          |      | Report numbers of outcome events or summary measures |      |      |
| Main results             | 16   |           |      |      |
| Page 10-11               |      |           |      |      |
|                          |      | (a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included |      |      |
|                          |      | (b) Report category boundaries when continuous variables were categorized |      |      |
|                          |      | (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period |      |      |
| Other analyses           | 17   |           |      |      |
| “N/A”                    |      | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses |      |      |
| Discussion               |      |           |      |      |
| Key results              | 18   |           |      |      |
| Page 14. Paragraph 3     |      | Summarise key results with reference to study objectives |      |      |
| Limitations              | 19   |           |      |      |
| Page 15. Paragraph 2     |      | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias |      |      |
| Interpretation           | 20   |           |      |      |
| Page 17. Paragraph 2     |      | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence |      |      |
| Page 18. Paragraph 2     |      |          |      |      |
| Generalisability         | 21   |           |      |      |
|                          |      | Discuss the generalisability (external validity) of the study results |      |      |
| Other information        |      |           |      |      |
| Funding                  | 22   |           |      |      |
| Declarations form        |      | Give the source of funding and the role of the funders for the present study and, if applicable, |      |      |
*Give information separately for exposed and unexposed groups.

Table 1. Main clinical characteristics of patients with celiac disease (CD), non-celiac gluten sensitivity (NCGS) and control.

|                        | CD (n=15) | NCGS (n=9) | Control (n=10) |
|------------------------|-----------|------------|----------------|
| Age (years)            |           |            |                |
| Mean ± SD              | 55.2 ± 15.9 | 49.8 ± 13.6 | 53.2 ± 9.1     |
| Female                 | 13        | 9          | 5              |
| Food allergies         | 2 *       | 1**        | 0              |
| Autoimmune disease     | 12        | 2          | 4              |
| Osteopenia/osteoporosis| 5         | 3          | 1              |
| Body mass index Kg/m^2 | 23.4 (21-39) | 21.5 (18-25) | 25 (22-28)     |

* Fish, ** Berries
Table 2. Serological markers (antibodies values are expressed as median with min-max) and the main laboratory variables (chemistries are expressed as the number of patients with abnormal values (%)).

|                  | CD (n=15)          | NCGS (n=9)        | Control (n=10)     |
|------------------|--------------------|-------------------|--------------------|
| DGP IgA (U/mL)   | 51 (12.3-132.3)    | 4.4 (3.5-19.6)    | 5.5 (4.2-36.6)     |
| DGP IgG (U/mL)   | 32 (4-143)         | 6.2 (3.9-63.1)    | 4.2 (3.2-9.5)      |
| tTg IgA (U/mL)   | 17 (2.3-436.1)     | 3.1 (0.6-15)      | 2.5 (1.5-4.9)      |
| EmA IgA positive | 11                 | 0                 | 0                  |
| HLA DQ2/DQ8 positive | 12*             | 6**               | NA                 |
| Hemoglobin < 13.0 g/dL | 5               | 0                 | 0                  |
| Ferritin < 11 ng/mL | 3               | 2                 | 0                  |
| Vitamin B12 < 180 pg/mL | 1            | 1                 | NA                 |
| Folates < 5.9 ng/mL | 6              | 1                 | NA                 |
| Vitamin D < 29 ng/mL | 11             | 3                 | NA                 |
| Vitamin D < 20 ng/mL | 5              | 2                 | NA                 |
| Albumin < 3.5 g/L | 1                 | 1                 | 0 (0.0)            |

*DGP IgA = IgA anti-deamidated gliadin antibodies DGP IgG= IgG anti-deamidated gliadin antibodies tTg IgA= IgA anti-transglutaminase antibodies EmA IgA= IgA anti-endomysium antibodies. NA= not-available
* HLA performed in 12 patients. ** HLA performed in 8 patients.

**Figures**
Figure 5. A-B

A  IL-4-expressing cells

B  TGF-β1-expressing cells

C  IL-10-expressing cells

Figure 5. C
Figure 1

Anti-inflammatory/pro-fibrogenic cytokines. (A, B, C, and D, left panel): Representative immunoperoxidase photomicrographs of control, non-celiac gluten sensitivity (NCGS) and celiac disease (CD). Arrows depict (A) IL-4, (B) TGF-β1, and (c) IL-10 immunoreactive cells. Original magnification was x320. (A, B, C, and D, right panel): Relative percentage expression of (A) IL-4, (B) TGF-β1, and (C) IL-10. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.
Figure 4. A-B

A  IL-17A-expressing cells

B  IL-21-expressing cells

C  IL-22-expressing cells

D  IL-23-expressing cells

Control (n=10)  NCGS (n=9)  CD (n=15)
Figure 2

Cytokines that induce and maintain Th17 polarization. (A, B, C, and D, left panel): Representative immunoperoxidase photomicrographs of control, non-celiac gluten sensitivity (NCGS) and celiac disease (CD). Arrows depict (A) IL-17A, (B) IL-21, (C) IL-22 and (D) IL-23 immunoreactive cells. Original magnification was x320. (A, B, C, and D, right panel): Relative percentage expression of (A) IL-17A, (B) IL-21, (C) IL-22 and (D) IL-23. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.
Figure 3

Cytokines that induce and maintain Th1 polarization. (A, B, C, and D, left panel): Representative immunoperoxidase photomicrographs of control, non-celiac gluten sensitivity (NCGS) and celiac disease (CD). Arrows depict (A) IL-2, (B) IL-12p40, (C) IL-15 and (D) IFN-γ immunoreactive cells. Original magnification was x320. (A, B, C, and D, right panel): Relative percentage expression of (a) IL-2, (b) IL-12p40, (c) IL-15 and (d) IFN-γ. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.
Figure 2. A-B

A

IL-1β-expressing cells

Control  NCGS  CD

![Image of IL-1β-expressing cells]

![Box plot showing immunoreactive cell percentage]

B

TNF-α-expressing cells

Control  NCGS  CD

![Image of TNF-α-expressing cells]

![Box plot showing immunoreactive cell percentage]

Figure 2. C-D

C

IL-6-expressing cells

Control  NCGS  CD

![Image of IL-6-expressing cells]

![Box plot showing immunoreactive cell percentage]

D

IL-8-expressing cells

Control  NCGS  CD

![Image of IL-8-expressing cells]

![Box plot showing immunoreactive cell percentage]
Figure 4

Pro-inflammatory cytokine-expressing cells in Celiac Disease. (A, B, C, and D, left panel): Representative immunoperoxidase photomicrographs of control, non-celiac gluten sensitivity (NCGS) and celiac disease (CD). Arrows depict (A) IL-1β, (B) TNF-α, (C) IL-6 and (D) IL-8 immunoreactive cells. Original magnification was x320. (A, B, C and D, right panel): Relative percentage expression of (A) IL-1β, (B) TNF-α, (C) IL-6 and (D) IL-8. Results are expressed as the mean (yellow line), median (black line), and 5th/95th percentiles.

Figure 5

Circulating and mucosal lymphocytes (mLs) in control, non-celiac gluten sensitivity (NCGS) and celiac disease (CD) groups. (A) Peripheral and (B) mLs CD4+ T cells; (C) Peripheral and (D) mLs CD4+/Foxp3+ Tregs; (E) Peripheral and (F) mLs CD4+/T-bet+ cells; (G) Peripheral and (H) mLs CD4+/IFN-γ+ cells; (I) Peripheral and (J) mLs CD4+/ROR-γt+ cells; (K) Peripheral and (L) mLs CD4+/IL-17A+ cells. Results are expressed as mean (black line) ± standard deviation. Control: n = 9, NCGS: n = 7 and CD: n = 7.