

Accepted Manuscript

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PII: S0016-5085(15)00767-2
DOI: [10.1053/j.gastro.2015.05.040](https://doi.org/10.1053/j.gastro.2015.05.040)
Reference: YGAST 59811

To appear in: *Gastroenterology*
Accepted Date: 20 May 2015

Please cite this article as: Carroccio A, D'Alcamo A, Cavataio F, Soresi M, Seidita A, Sciumè C, Geraci G, Iacono G, Mansueto P, High Proportions of People with Non-Celiac Wheat Sensitivity Have Autoimmune Disease or Anti-nuclear Antibodies, *Gastroenterology* (2015), doi: 10.1053/j.gastro.2015.05.040.

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High Proportions of People with Non-Celiac Wheat Sensitivity Have Autoimmune Disease or Anti-nuclear Antibodies

Short title: Risk of autoimmunity in NCWS

Antonio Carroccio (1,2), Alberto D'Alcamo (1), Francesca Cavataio (3), Maurizio Soresi (1), Aurelio Seidita (1), Carmelo Sciumè (4), Girolamo Geraci (4), Giuseppe Iacono (3), Pasquale Mansueto (1)

1) DiBiMIS University of Palermo, Italy

2) Internal Medicine, Giovanni Paolo II Hospital, Sciacca (ASP Agrigento), Italy

3) Pediatric Gastroenterology, ARNAS Di Cristina Hospital, Palermo, Italy

4) Surgery Department, University of Palermo, Italy

Funding/Support: The study was supported in part by the University of Palermo (grant 2012 ATE 0491 "Gluten sensitivity and IBS" and in part by the Italian Foundation for Celiac Disease (AIC) Grant for Project 013 2014.

Address for correspondence: Antonio Carroccio, Internal Medicine, via Ciaculli 207, 90124 Palermo, Italy. Fax number: +39 0925 84757 Mail: acarroccio@hotmail.com

Conflict of interest: All the Authors have no conflicts to declare.

Author Contributions:

Prof Carroccio had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Carroccio, Mansueto, D'Alcamo, Iacono.

Acquisition of data: Carroccio, D'Alcamo, Iacono, Cavataio, Seidita, Mansueto.

Endoscopy study: Sciumè, Geraci.

Analysis and interpretation of data: Carroccio, D'Alcamo, Soresi, Iacono, Cavataio, Mansueto.

Drafting of the manuscript: Carroccio, D'Alcamo, Mansueto.

Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: Soresi.

Acknowledgements

We wish to thank Ms. Carole Greenall BA MCIL for revising the text.

Abbreviations list:

AD: autoimmune diseases

ANA: anti-nuclear antibodies

anti-tTG: anti-transglutaminase

ATIs: amylase/trypsin inhibitors

CD: celiac disease

DBPC: double-blind placebo-controlled

EmA: anti-endomysium

FODMAP: dietary short chain carbohydrate

IBS: irritable bowel syndrome

NCWS: non-celiac wheat sensitivity

SD: standard deviation

Abstract:

Background & Aims: There is much interest in wheat sensitivity among people without celiac disease (NCWS), but little is known about any risks associated with the condition. We evaluated the prevalence of autoimmune diseases among patients with NCWS, and investigated whether they carry anti-nuclear antibodies (ANA).

Methods: We performed a retrospective study of 131 patients diagnosed with NCWS (121 female; mean age, 29.1 y) at 2 hospitals in Italy from January 2001 through June 2011. Data were also collected from 151 patients with celiac disease or irritable bowel syndrome (IBS) (controls). Patients' medical records were reviewed to identify those with autoimmune diseases. We also performed a prospective study of 42 patients (38 female; mean age, 34 y) diagnosed with NCWS from July 2011 through March 2014 at 3 hospitals in Italy. One hundred age- and sex-matched subjects with celiac disease or IBS served as controls. Serum samples were collected from all subjects and ANA levels were measured by immunofluorescence analysis. Participants completed a questionnaire and their medical records were reviewed to identify those with autoimmune diseases.

Results: In the retrospective analysis, similar portions of subjects with NCWS (29%) and celiac disease (29%) developed autoimmune diseases (mainly Hashimoto's thyroiditis, 29 cases), compared with a smaller proportion of subjects with IBS (4%) ($P<.001$). In the prospective study, 24% of subjects with NCWS, 20% of subjects with celiac disease, and 2% of subjects with IBS developed autoimmune diseases ($P<.001$). In the retrospective study, serum samples tested positive for ANA in 46% of subjects with NCWS (median titer 1:80), 24% of subjects with celiac disease ($P<.001$), and 2% of subjects IBS ($P<.001$); in the prospective study, serum samples were positive for ANA in 28% of subjects with NCWS, 7.5% of subjects with celiac disease ($P=.02$), and 6% of subjects with IBS ($P=.005$ vs patients with NCWS). ANA positivity was associated with the presence of the HLA DQ2/DQ8 haplotypes ($P<.001$).

Conclusions: Higher proportions of patients with NCWS or celiac disease develop autoimmune disorders, are ANA positive, and showed DQ2/DQ8 haplotypes compared to patients with IBS.

KEYWORDS: food allergy, gluten sensitivity, celiac disease, immune response

INTRODUCTION

Celiac disease (CD) is an immune-based reaction to dietary gluten (storage proteins found in wheat, barley, and rye) which primarily affects the small intestine in genetically predisposed patients and resolves when gluten is eliminated from the diet (1). Although it is not certain whether CD can be included among the autoimmune diseases, in CD patients circulating autoantibodies (anti-transglutaminase) are observed and a specific tissue cell type (enterocyte) is destroyed by CD8+ T cells (2). Furthermore, other autoimmune diseases have been reported in association with CD in 20-30% of cases (3,4).

In the last few years, a new clinical entity has emerged, which appears to include patients who consider themselves to be suffering from problems caused by wheat and/or gluten ingestion, even though they do not have CD or wheat allergy (5). This clinical condition has been named “Non-Celiac Gluten Sensitivity” (6), although in a recent paper we suggested the more appropriate term of “Non-Celiac Wheat Sensitivity” (NCWS) (7), since it is not known to date what component of wheat actually causes the symptoms. Other areas of doubt in NCWS regard its pathogenesis, as while some papers have reported intestinal immunologic activation (8-10), others have linked NCWS to the dietary short chain carbohydrate (FODMAP) load (11), thus ruling out an immunologic involvement in NCWS. In the present study we therefore compared the risk of “autoimmunity” in CD and NCWS patients, evaluating: a) the frequency of autoimmune diseases and b) the frequency of serum anti-nuclear antibody (ANA) positivity in these two conditions.

METHODS

Study design and population

The study design was divided into two different parts: a retrospective evaluation and a prospective survey. In the first, the clinical charts of NCWS patients attending the outpatient centers of the Department of Internal Medicine at the University Hospital of Palermo and the Department of Internal Medicine of the Hospital of Sciacca were reviewed with a retrospective method. They had all been diagnosed with NCWS between January 2001 and June 2011 and included in a previously published study (12). These charts included specific sections for associated autoimmune diseases and the presence of serum ANA. Incomplete clinical charts were excluded. Two control groups were selected. The first was composed of 101 CD patients and the second of 50 IBS patients. Both were randomly chosen by a computer-generated method from subjects diagnosed during the same period (2001-2011) and age- (± 2 years) and sex-matched ($\pm 5\%$) with the NCWS patients. The IBS controls had been

receiving the same elimination diet as the NCWS patients and had not shown any clinical improvement; they belonged to the cohort of subjects we had previously studied (12).

In the second part of the study, we prospectively surveyed adult patients with an irritable bowel syndrome (IBS)-like clinical presentation, according to the Rome II criteria, and a definitive diagnosis of NCWS. The patients were recruited between July 2011 and March 2014 at three centers: the same two mentioned above, together with the Gastroenterology Unit of the ARNAS Civico Hospital of Palermo, Italy. Most of the patients had been referred due to intestinal symptoms, whose onset, they reported, could be related to wheat ingestion. During the study period, the newly-diagnosed NCWS patients were randomly assigned to one of the several studies that we are currently performing on NCWS. In this way forty-two patients were recruited. Twenty-two of the patients included in the present study also accepted to be included in other, as yet unpublished studies. Two control groups were again selected. One included forty consecutive patients with a new CD diagnosis, sex- ($\pm 5\%$) and age-matched (± 2 years) with the NCWS patients, and enrolled during the prospective study period. The second group included fifty subjects with IBS unrelated to NCWS or other types of food “intolerance”, who were consecutively recruited during the study period and sex- and age-matched with the NCWS patients. IBS diagnosis had been made in accordance with the Rome II criteria and none of these subjects improved on an elimination diet without wheat, cow’s milk, egg, tomato or chocolate.

For both the prospective and the retrospective studies, exclusion criteria were: a) positive EmA in the culture medium of the duodenal biopsies, even if the villi/crypts ratio in the duodenal mucosa was normal; b) self-exclusion of wheat from the diet and refusal to reintroduce it, before entering the study; c) other “organic” gastrointestinal diseases.

NCWS diagnosis:

To diagnose NCWS in both the retrospective and the prospective parts, various criteria were adopted. Firstly, all the patients met the recently proposed criteria (13): a) negative serum anti-transglutaminase (anti-tTG) and anti-endomysium (EmA) IgA and IgG antibodies; b) absence of intestinal villous atrophy; c) IgE-mediated immune-allergy tests negative to wheat (skin prick tests and/or serum specific IgE detection). Other criteria adopted in our patients were: 1) resolution of the IBS symptoms on a standard elimination diet without wheat, cow’s milk, egg, tomato, chocolate or other food(s) causing self-reported symptoms; 2) symptom reappearance on double-blind placebo-controlled (DBPC) wheat challenge, performed as previously described (12). As in previous studies,

DBPC cow's milk protein challenge and other "open" food challenges were also performed (12) (for details see Supplemental File 1). Further inclusion criteria were: a) age >18 years; b) follow-up duration longer than nine months after the initial diagnosis; c) at least two outpatient visits during the follow-up period.

CD diagnosis

CD was diagnosed in the presence of positive serum anti-tTG and/or EmA antibodies and of duodenal villous atrophy at histological examination, followed by symptom resolution after commencement of the gluten-free diet.

Outcomes

Serum anti-nuclear antibodies (ANA)

The frequency of serum ANA positivity in NCWS and the control groups was evaluated. ANA was identified by HEp-2 cells, using an indirect immunofluorescence technique; a titer of 1:40 or higher was considered positive and the sera were titrated at progressive dilutions until they became negative. In the patients included in the prospective study, the ANA pattern was also classified as "homogeneous", "fine speckled" or "coarse speckled" and "nucleolar".

Frequency of associated autoimmune diseases (AD)

The clinical chart used for the retrospective study listed the autoimmune diseases and physicians had to fill out the specific fields. The hospital records of all patients diagnosed with an autoimmune disorder were thoroughly examined to ascertain whether the recognized diagnostic criteria for each disorder had been fulfilled.

Questionnaire for autoimmunity

In the prospective study, the presence of autoimmune disorders both in the study and control groups was evaluated by a structured questionnaire and a review of patients' clinical records. The presence of one of the following was looked for in all subjects: connective tissue diseases (rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, scleroderma, mixed connective tissue disease, ankylosing spondylitis, or Sjögren's disease), autoimmune endocrinological diseases (Hashimoto's thyroiditis, Graves' disease, insulin-dependent diabetes mellitus, Addison's disease), autoimmune hepatitis (Types 1, 2 and 3), primary biliary cirrhosis, epilepsy with cerebral calcification, unexplained cerebellar ataxia, alopecia, psoriasis, atrophic autoimmune gastritis, and immune anemia,

neutropenia, or thrombocytopenia. Age at diagnosis of the diseases and treatments received were recorded in all cases.

Laboratory Methods

Serology for CD, duodenal histology studies and HLA-DQ typing were performed in all patients as previously described (12) (see Supplemental File 1). Duodenal histology lesions were classified according to Corazza and Villanacci as: normal mucosa (villi/crypts ratio >3 and CD3+ IEL $< 25/100$ enterocytes), Grade A (villi/crypts ratio >3 and CD3+ IEL $> 25/100$ enterocytes), Grade B1 (villi/crypts ratio <3 - partial villous atrophy) and Grade B2 (total villous atrophy) (14).

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD) when the distribution was Gaussian and differences were calculated using Student's *t*-test. Otherwise, data were expressed as median and range, and analyzed with the Mann–Whitney U test. Fisher's exact or the chi-square tests were used where appropriate. The Mantel-Haenszel test was used to compare the severity of the duodenal histology damage in NCWS and CD patients. Multiple logistic regression analysis was performed to evaluate the association between the presence of serum ANA and the other clinical variables evaluated. $P < 0.05$ was considered significant. All analyses were performed using the SPSS software package (version 16.0; Chicago, IL, USA).

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, was approved by the institution's human research committee (University Hospital of Palermo) and registered at clinicaltrials.gov (registration number: NCT02248545).

RESULTS

Patient clinical characteristics

After the exclusion of the incomplete clinical records in which some data were lacking and of those of the patients who had tested positive for anti-endomysium antibodies in the culture of the duodenal mucosa, one hundred and thirty-one NCWS patients of the retrospective cohort were included (see Supplemental File 2). Tables 1 and 2 show the clinical characteristics of the patients included in the retrospective and in the prospective studies respectively, in comparison with the control groups. In general, in NCWS patients there was a higher percentage of self-reported wheat intolerance and coexisting atopic diseases than CD controls ($P < 0.001$).

Frequency of positive serum ANA

Patients with NCWS were more likely to be ANA positive than both patients with celiac disease and IBS patients, in both the retrospective and prospective studies (Figure 1). In the retrospective study, NCWS patients showed a very high frequency of serum ANA (46%), significantly higher than in CD (24%) subjects and IBS (2%) controls ($P < 0.001$, for both). Median values of the ANA titer were 1:80 both in NCWS (range 1:40-1:640) and in CD (range 1:40-1:1280).

These results were confirmed in the prospective study, where the frequency of ANA positivity was higher in NCWS patients (28%) than in the two control groups (7.5% in CD, $P < 0.02$, and 6% in IBS patients, $P < 0.005$). The ANA titer ranged between 1:40 and 1:1280 in both groups. The ANA pattern in the prospective study was equally distributed: “homogeneous” in 11 cases, “fine speckled” in 11 cases, “coarse speckled” in 9 cases, and “nucleolar” in 11 cases.

Variables associated to serum ANA positivity

The presence of serum ANA in NCWS patients correlated with HLA DQ2/DQ8 haplotypes. In detail, in the retrospective study, HLA DQ2/DQ8 haplotypes were present in 49 / 59 ANA positive versus 16 / 72 ANA negative patients; $P < .001$. A significant correlation was also observed with a high frequency of duodenal lymphocytosis ($P = .02$) (Figure 2). No correlation was found with sex, age, disease duration, presence of coexisting atopic diseases, multiple food sensitivity, anemia or BMI < 20 . On multivariate analysis the only variable significantly associated with ANA positivity was the presence of the HLA DQ2/DQ8 haplotypes (OR=3.5, 95% CI 1.6-5.8, $P < .001$).

The prospective study confirmed the association between ANA positivity and HLA status: HLA DQ2/DQ8 haplotypes were present in 10 / 12 ANA positive versus 13 / 30 ANA negative patients ($P = 0.04$). Again, no other variables correlated with ANA positivity, including duodenal lymphocytosis.

Frequency of associated autoimmune diseases (AD)

Patients with NCWS showed a frequency of AD similar to CD but significantly higher than IBS controls, in both the retrospective and prospective studies (Figure 1). In the retrospective study, NCWS patients had an associated AD in 29% of the cases versus 21% in CD patients, and both these groups had a higher frequency than IBS controls (NCWS vs IBS $P < 0.001$; CD vs IBS $P < 0.01$). In detail, NCWS patients presented one or more of the following diseases: Hashimoto's thyroiditis (29 cases), psoriasis (4 cases), Type 1 diabetes (4 cases), mixed connective tissue disease (1 case), ankylosing spondylitis (1 case).

In the prospective study, the frequency of AD was also virtually identical to that of the retrospective study. Ten NCWS patients showed concomitant autoimmune thyroiditis (24%), but no other autoimmune diseases were found.

In all NCWS patients, the diagnosis of thyroiditis had been made before that of NCWS (median time 8 years). Eight of the ten patients with NCWS and thyroiditis presented hypothyroidism and were receiving replacement therapy.

Variables associated to the presence of autoimmune diseases (AD)

Different trends in the presence of autoimmune diseases were observed in the two study parts.

In the retrospective study, their presence in NCWS patients did not correlate with any of the other variables studied, including the presence of haplotypes DQ2/DQ8, which were present in 19 / 38 patients (50%) with autoimmune diseases and in 40 / 93 (43%) NCWS patients without an associated autoimmune disease. However, in the prospective study there was a trend towards an association with thyroiditis in NCWS patients and the presence of the haplotypes DQ2/DQ8 in 8 / 10 patients (80%) with thyroiditis and in 15 / 32 patients (47%) without thyroiditis, but this association was not statistically significant ($P=0.08$).

As regards the possible association between serum ANA positivity and the presence of autoimmune diseases, different results were also found. In the retrospective study, serum ANA were present in 20 / 38 (53%) patients with autoimmune diseases and in 39 / 93 without (42%) ($P=0.33$); in contrast, in the prospective study ANA positivity was associated with thyroiditis: 6 / 10 with versus 6 / 32 without thyroiditis ($P=0.02$).

No other variables significantly correlated with the presence of autoimmune diseases in either of the two study parts, including duodenal lymphocytosis, which was present only in 15 of the 38 NCWS patients with AD in the retrospective study and in 4 of the 10 with AD in the prospective study.

DISCUSSION

NCWS is an emerging clinical condition which, in the last few years, has attracted the interest of researchers (6,13). Several data, including the present ones, seem to indicate that it is a sex-related disease, with a much higher frequency in females (9,12,14). However, the lack of a diagnostic marker for NCWS is the main problem in identifying patients. Furthermore, after basing the diagnosis on DBPC challenge, many clinical characteristics still remain to be defined, including whether NCWS is

associated to nutritional problems or autoimmune diseases, as is the case for CD. Recent data have demonstrated that nutrient deficiency is present in about 18% of NCWS patients (15) and we showed that a low body mass index and reduced bone mass density are frequent in these patients (16). Data about autoimmunity in NCWS are very scarce, no past studies have been specifically designed to clarify this aspect, and, in particular, no previous investigations have studied the presence of serum autoantibodies.

We therefore retrospectively evaluated the frequency of serum ANA positivity in NCWS patients diagnosed by DBPC challenge in a historical cohort studied in the years 2001-11. Serum ANA were positive in 46% of the NCWS patients, a percentage significantly higher than those observed in the celiac (24%) and IBS controls (2%). Moreover, the findings obtained in the prospective cohort, although collected in a small group of NCWS patients, also confirmed that NCWS is characterized by a higher frequency of positive serum ANA than in CD (28% vs. 7.5%). Literature data have reported a frequency of positive autoantibodies in CD patients of about 25%, although the specific frequency of ANA positivity was about 8% (17,18). The data in healthy controls, using the IF method, have shown ANA positivity in a relatively high proportion of individuals: more than 10% at 1:80 dilution (19,20). However, although the reported frequency of positive serum ANA in the general population was high, the frequency we observed in the NCWS was evidently even higher. We also found ANA positive sera at a low dilution (median 1:80) and in the absence of specific signs/symptoms of overt autoimmune diseases, in particular systemic lupus erythematosus.

ANA positivity in our patients was found to be associated with the presence of the DQ2/DQ8 haplotypes and - to a lesser extent - with the presence of duodenal lymphocytosis (Grade A histology). Obviously, these associations strongly suggest a celiac condition, but it must be underlined that all the patients we included were negative for CD specific antibodies and showed normal intestinal villi when evaluated after a prolonged time on a gluten-containing diet (at least 100 grams of wheat per day, for a minimum of four weeks). Furthermore, we also excluded patients, both from the retrospective and from the prospective cohorts, who tested positive for anti-endomysium antibodies in the culture medium of the duodenal biopsies, a positive result which could precede the onset of overt CD (21). However, it has been reported that analysis of the IEL flow cytometric pattern is an accurate method for identifying CD in the initial diagnostic biopsy of seronegative patients presenting with lymphocytic enteritis and it seems to be better than anti-TG2 intestinal deposits (22). Thus, a more accurate evaluation of the duodenal histology, using the above method, should be

recommended in all the NCWS patients who show the DQ2 or the DQ8 haplotypes and duodenal lymphocytosis.

We also found a quite high frequency of autoimmune thyroiditis associated to NCWS in both cohorts (22% and 24% in the retrospective and in the prospective cohorts, respectively), although other autoimmune diseases - more frequently psoriasis and Type 1 diabetes mellitus - were more rarely reported in the retrospective cohort. Previous studies have reported conflicting data about the presence of autoimmune comorbidities in NCWS patients. These were not reported in two studies involving a relatively low number of patients (23, 24), but a recent multicenter Italian study including nearly five hundred patients reported a high prevalence of Hashimoto's thyroiditis (25) and the same study showed that other autoimmune conditions such as psoriasis can coexist in these patients, as previously reported in a clinical setting recalling NCWS (26). In this respect, our study design with a structured questionnaire and based on a review of patients' clinical records, both dedicated to ascertain the presence of autoimmune comorbidities, showed that NCWS might behave in a similar way to CD (3,4).

Once again, these findings pose the problem of whether there is a real distinction between NCWS and CD and suggest a possible overlap between these two conditions. However, it should be emphasized that the majority of the autoimmune diseases were found in patients with absolutely normal duodenal histology and this is hard evidence that NCWS is an immune-mediated condition different from CD. Another strong immunologic hypothesis to consider is the one suggested by Junker and coll. who identified the amylase/trypsin inhibitors (ATIs) as activators of innate immune responses by engaging the toll-like receptor system (27). In fact, it has been demonstrated that patients with hereditary angioedema tend to produce autoantibodies and have a propensity to develop immunoregulatory disorders, probably due to the increased activation of B cells, which was found to be associated with a high expression of TLR-9 (28). Whether similar mechanisms have a role in NCWS autoimmunity obviously remains to be demonstrated.

In spite of the interesting results, however, the limits of our study must be underlined. We studied patients referred to tertiary centers with experience in CD and NCWS and this factor clearly lead to a selection bias, therefore our results cannot be extended to the broad population of self-treated or to diagnosed NCWS patients. Furthermore, in this respect, we and others have suggested that NCWS cannot be considered a unique condition but rather an "umbrella term" which includes various conditions with different types of pathogenesis (12, 29-33). As regards our previous hypothesis that

NCWS could be an allergic disease (28), it should also be remembered that negative IgE test results cannot exclude food allergy. In the absence of specific biomarkers, we should cautiously indicate that this high frequency of autoimmunity can apply to NCWS patients who react to a challenge with a low amount of wheat (equivalent to about 20 grams of bread in our experiment). Finally, it is noteworthy that of the 273 patients included in the original cohort of the retrospective study, 123 (44%) were excluded as the data on their charts concerning a possible associated condition secondary to autoimmunity were incomplete. We cannot rule out the possibility that if the clinical records had been complete they would have included patients with clinical characteristics pointing to associated autoimmune features.

The strong points of our data, on the other hand, were the patient selection (based on a NCWS diagnosis made by using the DBPC challenge method), the study design, (which was specifically constructed to reveal the presence of autoimmune diseases and the frequency of serum ANA antibody positivity) and the confirmation of the retrospective results by the prospective ones.

In conclusion, our data showed a strong tendency towards autoimmunity in the NCWS patients, characterized by both associated autoimmune diseases and the presence of serum ANA positivity, which in turn was correlated with the HLA DQ2/DQ8 haplotypes.

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Author names in bold designate shared co-first authorship.

Legend of Figure

FIGURE 1: Frequency of HLA haplotypes DQ2 and/or DQ8, positive serum anti-nuclear antibodies (ANA) and autoimmune diseases in 131 non-celiac wheat-sensitive (NCWS) patients, 101 celiac disease (CD) controls and 50 irritable bowel syndrome (IBS) controls, included in the retrospective study (PANEL A) and in 42 non-celiac wheat-sensitive (NCWS) patients, 40 celiac disease (CD) controls and 50 irritable bowel syndrome (IBS) controls, included in the prospective study (PANEL B).

Footnotes:

PANEL A:

Serum ANA positivity: NCWS vs. CD $P < 0.001$; NCWS vs. IBS $P < 0.001$; CD vs. IBS $P < 0.001$

Frequency of autoimmune diseases: NCWS vs. CD $P = 0.17$; NCWS vs. IBS $P < 0.001$; CD vs. IBS $P = 0.01$

PANEL B:

Serum ANA positivity: NCWS vs. CD $P = 0.02$; NCWS vs. IBS $P = 0.005$; CD vs. IBS $P = 0.68$

Frequency of autoimmune diseases: NCWS vs. CD $P = 0.8$; NCWS vs. IBS $P = 0.002$; CD vs. IBS $P = 0.01$

FIGURE 2: Percentage of DQ2/DQ8 positive patients and of grade A duodenal histology (normal villi/crypts ratio with Intra Epithelial Lymphocytes $> 25 / 100$ enterocytes) in NCWS patients showing serum ANA positivity (59 cases) or serum ANA negativity (72 cases).

Table 1: Clinical characteristics of 131 non-celiac wheat sensitive (NCWS) patients, 101 celiac disease (CD) controls and 50 irritable bowel syndrome (IBS) controls, included in the retrospective study.

	NCWS (n=131)	CD (n=101)	IBS (n=50)	P value
Age (X $\pm$ SD)	39.1 $\pm$ 11	40.1 $\pm$ 12.3	38.8 $\pm$ 10.8	Not Applicable, matching factor
Sex	121 F / 10 M	91 F / 10 M	44 F / 6 M	Not Applicable, matching factor
Median (range) duration of symptoms (years)	6.5 (1-40)	5.5 (1-30)	6 (1-25)	NCWS vs. CD P=0.8 NCWS vs. IBS P=0.8 CD vs. IBS P=0.8
Self-reported wheat intolerance	78/131 (59%)	20/101 (20%)	8/50 (16%)	NCWS vs. CD P< 0.001 NCWS vs. IBS P<0.001 CD vs. IBS P=0.6
Family history of CD	10/131 (8%)	17/101 (17%)	0/50	NCWS vs. CD P=0.04 NCWS vs. IBSP=0.06 CD vs. IBS P=0.01
Coexisting atopic diseases	39/131 (30%)	10/101 (10%)	3/50 (6%)	NCWS vs. CD P < 0.001 NCWS vs. IBS P< 0.001 CD vs. IBS P=0.5
Body Mass Index < 20	32/131 (24%)	26/101 (26%)	4/50 (8%)	NCWS vs. CD P=0.9 NCWS vs. IBS P=0.02 CD vs. IBS P=0.02
Anemia	34/131(27%)	52/101 (52%)	3/50 (6%)	NCWS vs. CD P<0.001 NCWS vs. IBS P<0.001 CD vs. IBS P<0.001
Multiple food sensitivity	90/131 (69%)	not evaluated	not evaluated	
Haplotypes DQ2 or DQ8	65/131 (50%)	101/101 (100%)	14/50 (28%)	NCWS vs. CD P<0.001 NCWS vs. IBS P< 0.001 CD vs. IBS P<0.001

	NCWS (n=131)	CD (n=101)	IBS (n=50)	P value
Duodenal histology			not evaluated	
Normal	26 (20%)	0		P<0.001
Grade A	105 (80%)	0		
Grade B1	0	64 (64%)		
Grade B2	0	37 (36%)		

Footnotes: CD and IBS controls were randomly chosen by a computer-generated method from subjects diagnosed during the same period and age- and sex-matched (+ 5%) with the NCWS patients.

Family history of CD was evaluated in first degree family members.

Anemia was defined by Hb values <12 g/dl in females and <13 g/dl in males

Duodenal histology lesions were classified according to Corazza and Villanacci as: normal mucosa (villi/crypts ratio >3 and CD3+ IEL < 25/100 enterocytes), Grade A (villi/crypts ratio >3 and CD3+ IEL > 25/100 enterocytes), Grade B1 (villi/crypts ratio <3 - partial villous atrophy) and Grade B2 (total villous atrophy)

Table 2: Clinical characteristics of 42 non-celiac wheat sensitive (NCWS) patients, 40 celiac disease (CD) controls and 50 irritable bowel syndrome (IBS) controls, included in the prospective study

	NCWS (n=42)	CD (n=40)	IBS (n=50)	P value
Age (X $\pm$ SD)	34 $\pm$ 12	35.5 $\pm$ 11.3	33.1 $\pm$ 7.6	Not Applicable, matching factor
Sex	38 F / 4 M	35 F / 5 M	43 F / 7 M	Not Applicable, matching factor
Median (range) duration of symptoms (years)	5.3 (1-35)	5.0 (1-20)	5.4 (1-28)	NCWS vs. CD P= 0.77 NCWS vs. IBS P=0.79 CD vs. IBS P=0.81
Self-reported wheat intolerance	30/42 (71%)	9/40 (22%)	8/50 (16%)	NCWS vs. CD P< 0.001 NCWS vs. IBS P< 0.001 CD vs. IBS P=0.43
Family history of CD	8/42 (19%)	6/40 (15%)	0/50	NCWS vs. CD P=0.77 NCWS vs. IBS P= 0.01 CD vs. IBS P= 0.01
Co-existing atopic diseases	14/42 (33%)	6/40 (15%)	4/50 (8%)	NCWS vs. CD P=0.07 NCWS vs. IBS P= 0.01 CD vs. IBS P=0.32
Body Mass Index < 20	10/42 (24%)	10/40 (25%)	4/50 (8%)	NCWS vs. CD P=1 NCWS vs. IBS P= 0.05 CD vs. IBS P = 0.04
Anemia	14/42 (33%)	16/40 (40%)	3/50 (6%)	NCWS vs. CD P=0.6 NCWS vs. IBS P< 0.001 CD vs. IBS P< 0.001
Multiple food sensitivity	18/42 (43%)	not evaluated	not evaluated	
Haplotypes DQ2 or DQ8	23/42 (55%)	40/40 (100%)	17/50 (34%)	NCWS vs. CD P<0.001 NCWS vs. IBS P= 0.05 CD vs. IBS P<0.001

	NCWS (n=42)	CD (n=40)	IBS (n=50)	P value
Duodenal histology			not evaluated	P< 0.001
Normal	17 (41%)	0		
Grade A	25 (59%)	0		
Grade B1	0	28 (70%)		
Grade B2	0	12 (30%)		

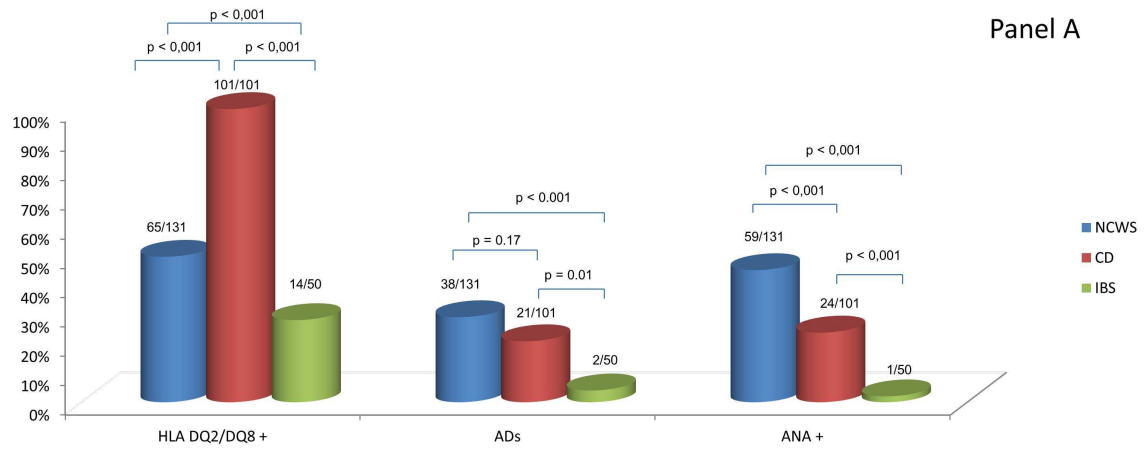
Footnotes: CD and IBS controls were randomly chosen by a computer-generated method from subjects diagnosed during the same period, and age- and sex-matched (+ 5%) with the NCWS patients.

Family history of CD was evaluated in first-degree family members.

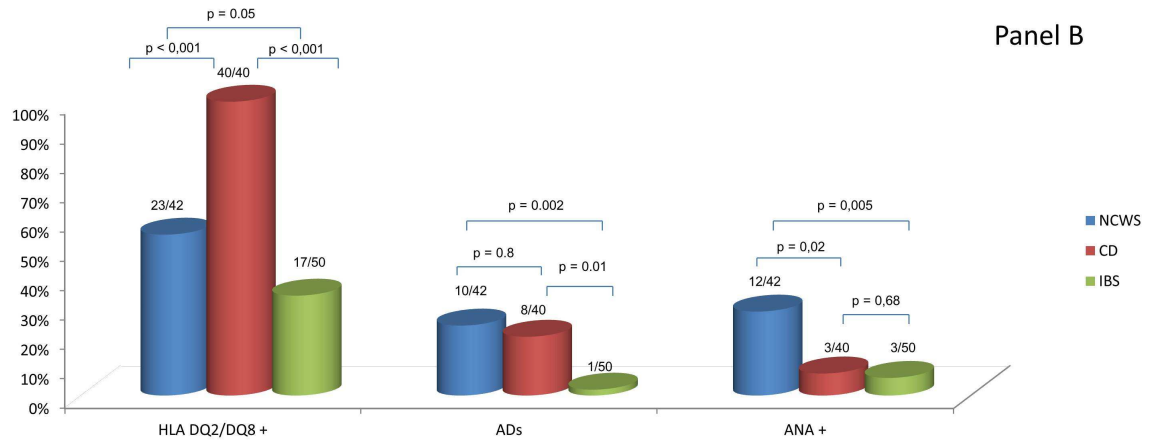
Anemia was defined by Hb values <12 g/dl in females and <13 g/dl in males

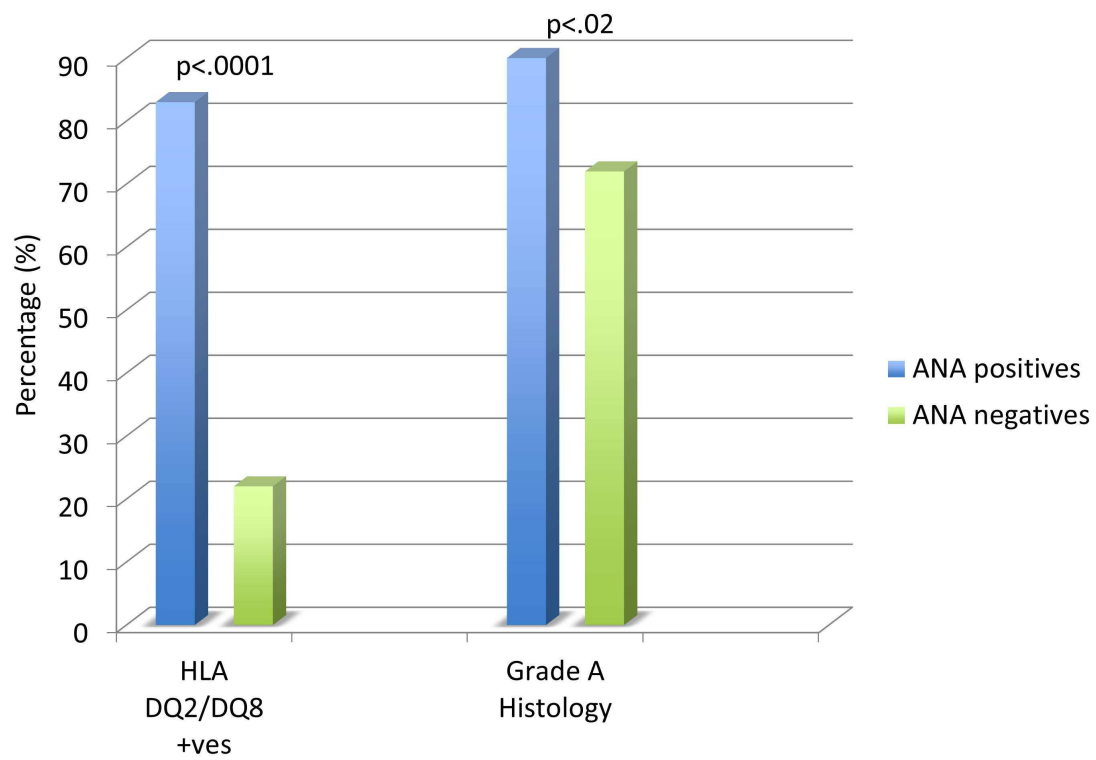
Duodenal histology lesions were classified according to Corazza and Villanacci as: normal mucosa (villi/crypts ratio >3 and CD3+ IEL < 25/100 enterocytes), Grade A (villi/crypts ratio >3 and CD3+ IEL > 25/100 enterocytes), Grade B1 (villi/crypts ratio <3 - partial villous atrophy) and Grade B2 (total villous atrophy)

Panel A



Panel B





Elimination diet and double-blind placebo-controlled (DBPC) challenge

On entering the study all patients commenced a standard elimination diet, with the exclusion of wheat, cow's milk, eggs, tomato and chocolate. Patients self-reporting food hypersensitivity were also asked to avoid ingestion and/or contact with the food(s) causing symptoms. Food diaries were maintained by the patients during the elimination diet period to assess dietary intake and adherence to the diet. After four weeks on elimination diet, they underwent DBPC challenges. The challenges were performed with the reintroduction of a single food at a time. Patients were randomized to receive either the "active food" or the placebo, according to a computer-generated order, determined by an observer not involved in the study.

In the case of wheat, the DBPC challenge was performed with capsules coded A or B containing wheat or xylose, respectively. Capsules A or B were given for 2 consecutive weeks and then after 1 week of washout the patients received the other capsules for another 2 weeks (cross-over design). Wheat challenge was performed administering a daily dose of 13g of flour, equal to about 20g of bread. A total of 12 capsules daily were given three times daily, away from meals.

DBPC for cow's milk was performed by administering capsules coded as A or B containing milk proteins (casein from bovine milk, lactoalbumin, lactoglobulin - daily dose 6g, equal to about 200ml of cow's milk) or xylose, respectively. A total of 6 capsules daily were given three times daily, away from meals.

The codes of the capsules were broken only at the end of the study and the investigators did not know the content of the capsules during the study period. Challenges for other foods in patients with suspected multiple food hypersensitivity were performed in an open fashion.

During all phases of the study, including the challenge period, the severity of symptoms was recorded: the patients completed a 100mm visual analog scale, with 0 representing no symptoms, which assessed overall symptoms and the specific symptoms they each reported.

The challenges were stopped when clinical reactions occurred (increase in VAS score >30) for at least two consecutive days (onset of abdominal discomfort or pain, associated with a change in stool frequency and/or stool appearance). The challenges were considered positive if the same symptoms which had been initially present reappeared after their disappearance on elimination diet.

Laboratory methods for CD diagnosis

On entering the study all patients underwent serum anti-tTG IgA and IgG, EmA IgA, and anti-gliadin (AGA) IgA and IgG assays, performed using commercial kits (Eu-tTG IgA, anti-endomysium, and anti-gliadin IgA and IgG, Eurospital Pharma; Trieste, Italy). Patients were also typed for HLA-DQ phenotypes by polymerase chain reaction using sequence-specific primers, with a rapid method (DQ-CD Typing Plus by BioDiaGene, Palermo, Italy)

Number of pts included/excluded
in the retrospective study

