Randomized Double-Blind Placebo-Controlled Crossover Trial for the Diagnosis of Non-Celiac Gluten Sensitivity in Children

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OBJECTIVES: **Non-celiac gluten sensitivity (NCGS) is characterized by intestinal and extra-intestinal symptoms that are related to the ingestion of gluten in subjects who are not affected by either celiac disease (CD) or wheat allergy (WA). In this multicenter study, we aim for the first time to evaluate the prevalence of NCGS in pediatric subjects with chronic functional gastrointestinal symptoms associated with gluten ingestion using a double-blind placebo-controlled (DBPC) gluten challenge with crossover.**

METHODS: **Among 1,114 children with chronic gastrointestinal symptoms (negative CD and WA), those exhibiting a positive correlation between symptoms and gluten ingestion were eligible for a diagnostic challenge including the following phases: run-in, open gluten-free diet (GFD) and DBPC crossover gluten challenge. Patients were randomized to gluten (10 g/daily) and placebo (rice starch) for 2 weeks each, separated by a washout week. The gluten challenge was considered positive in the presence of a minimum 30% decrease of global visual analogue scale between gluten and placebo.**

RESULTS: **Out of 1,114 children, 96.7% did not exhibit any correlation with gluten ingestion. Thirty-six children were eligible; after the run-in and open GFD, 28 patients entered the gluten challenge. Eleven children (39.2%; 95% CI: 23.6–53.6%) tested positive.**

CONCLUSIONS: **This is the first demonstration of the existence of NCGS in children that reinforce the need for a DBPC for the diagnosis as the diagnosis is ruled out in >60% of cases. The registration identifier in ClinicalsTrials.gov is NCT02431585.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

Non-celiac gluten sensitivity (NCGS) is characterized by intestinal and extra-intestinal symptoms that are related to the ingestion of gluten in subjects who are not affected by either celiac disease (CD) or wheat allergy (WA) (1). NCGS is receiving widespread interest from the general public and mass media and an increasing number of patients often embark on a self-administered gluten-free diet (GFD) without any medical indications. Consequently, correct diagnosis is necessary to appropriately manage these patients and to avoid useless and costly diets. In the last consensus on NCGS, the double-blind placebo-controlled (DBPC) challenge is indicated as the gold standard for diagnosis (2). In adults, several DBPC studies support the existence of this entity (3–6), and we were the first to report a pediatric series of NCGS (7); however, to the best of our knowledge, no DBPC studies have been performed in children so far.

The need to define the dimension of the problem stems from the observation that GFD is becoming popular even at pediatric age. It has been reported that ~5% of children and adolescents in New Zealand avoid gluten, although only 1% had a CD diagnosis

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(8). These children usually complain of functional gastrointestinal disorders (FGIDs) that are not responsive to common treatments.

FGIDs have a significant impact in pediatric clinical practice, account from 2% to 4% of all general pediatric office visits, and are associated with multiple comorbidities, poor quality of life, and school absenteeism (9,10). In a third of patients with FGIDs, pain persists even for >5 years, despite frequent medical attention (11). The pathogenesis underlying FGIDs remains unclear (12); altered gut motility, visceral hypersensitivity, abnormal brain–gut interaction, psychosocial disturbance and immune activation have been suggested as possible explanations for the symptoms (13,14). The role of dietary factors in the pathogenesis of FGIDs, mainly for irritable bowel syndrome (IBS), has been a topic of great scientific interest in the last decade. In particular, several studies in adults have demonstrated the possibility of a causal relationship between the ingestion of gluten and the development of symptoms in the absence of CD and WA (3–6).

In this multicenter study, for the first time in the pediatric literature, we aimed to evaluate the prevalence of NCGS in children with chronic functional gastrointestinal symptoms associated with gluten ingestion using a DBPC gluten challenge with crossover.

PATIENTS AND METHODS

This paper reports a multicenter prospective DBPC gluten challenge with crossover performed in five pediatric centers in Italy between 2013 and 2016, promoted and coordinated by the Pediatric Gastroenterological Center of the University of Bari. Enrolling centers joined the study at different times: Santa Maria del Carmine Hospital of Rovereto (TN) (2013), Santa Maria Incoronata dell'Olmo Hospital (Cava de Tirreni–Salerno) (2014), S. Paolo Hospital-University of Milan (2014), Catholic University (Rome) (2015).

The study comprised two steps: the first aimed to assess the presence of any possible correlation of symptoms with gluten ingestion throughout a detailed clinical/dietetic history to select children eligible for the second diagnostic step. Therefore, children with a positive history of functional chronic gastrointestinal symptoms (chronic abdominal pain, diarrhea, bloating, dyspeptic symptoms) with or without extra-intestinal manifestations to be eligible had to (a) report a correlation of symptoms with gluten ingestion and (b) test repeatedly negative (at least twice) for CD serology while on gluten containing diet (IgA-endomysial and IgA-tissue transglutaminase antibodies) food-specific-IgE (gluten and wheat), skin prick tests, prick by prick, and atopy patch tests to wheat. Based on Rome III criteria, in presence of alarm symptoms, children were investigated as appropriate in order to exclude organic conditions.

Before entering the diagnostic step, all children underwent the following tests: (a) antigliadin-antibodies-IgA (if not previously determined; ORGENTEC Diagnostika; Mainz, Deutschland); (b) hematological parameters including hemoglobin, serum iron, ferritin, aspartate aminotransferase, and erythrocyte sedimentation rate; (c) human leucocyte antigen class II typing (DQ-CD Typing Plus; DiaGene, Palermo, Italy); (d) for ethical reasons, duodenal biopsy was offered on voluntary basis only in patient with human leucocyte antigen DQ 2/8.

The diagnostic step included three phases (run-in, gluten elimination diet, gluten challenge), during which all children were asked to complete a global daily visual analogue scale (VAS) corresponding to their perception of gastrointestinal symptoms and weekly questionnaires to assess symptoms and quality of life (IBS–SS (Irritable bowel syndrome–severity score), mBSFS-C (Bristol Stool Form Scale for Children), and STAIC (State-Trait Anxiety Inventory for Children)).

The run-in phase (phase I: weeks 1–2) aimed to assess basal symptoms while on a gluten-containing diet; only symptomatic children progressed to the open diagnostic gluten-elimination diet (phase II: week 3–4). In the presence of a VAS score reduction >30% while on GFD (GFD-responsive), the gluten challenge phase (phase III: week 5–9) was offered.

Gluten challenge

While on a strict GFD, patients were randomized to take gluten or placebo for 14 days; then, the children from both arms started the wash-out period of one week and subsequently started the final 2-week period on placebo or gluten sachets, depending on a computer-generated randomization list. The design of the diagnostic step is described in **Figure 1**.

Gluten or placebo was administered as one sachet per day. The gluten used contained 80% protein; the non-protein part was mainly made of starch (14%), fiber (2%), fat (1.5%), and ash (0.75%). Moreover, as suggested by Salerno criteria our preparation contained ~0.4 g of ATIs per sachets. Each sachet contained either 10 g of gluten or placebo (indistinguishable in appearance and texture), and parents were instructed to disperse the content in any food (avoiding water/fruit juice/milk) administered during the day. Sachets containing gluten had the same shape, dimension, indication, and appearance as those containing placebo and were marked with a serial number. The manufacturer that provided this material was independent of the study (MOVISCOM SpA, Rome, Italy), and to ensure blindness of the investigators and patients, only the manufacturers were in a position to associate each sachet number with its content. Rice starch was chosen as the placebo to avoid FODMAP (Fermentable Oligosaccharides Disaccharides Monosaccharides And Polyols)-containing cornstarch. To assess compliance, patients were interviewed on a regular basis by medical personnel. Compliance was calculated as the percentage of returned sachets and ingested study product: a rate >80% was set as the minimum for both.

Dietetic follow-up

Throughout the trial an experienced dietitian (SD) performed the dietetic follow-up. Before and during the trial, she instructed all the families to guarantee a well-balanced diet with proper gluten avoidance without modification to the dietetic habits.

Questionnaires

According with the Salerno Criteria (2), to assess symptom severity, all children completed a combination of the self-reporting VAS

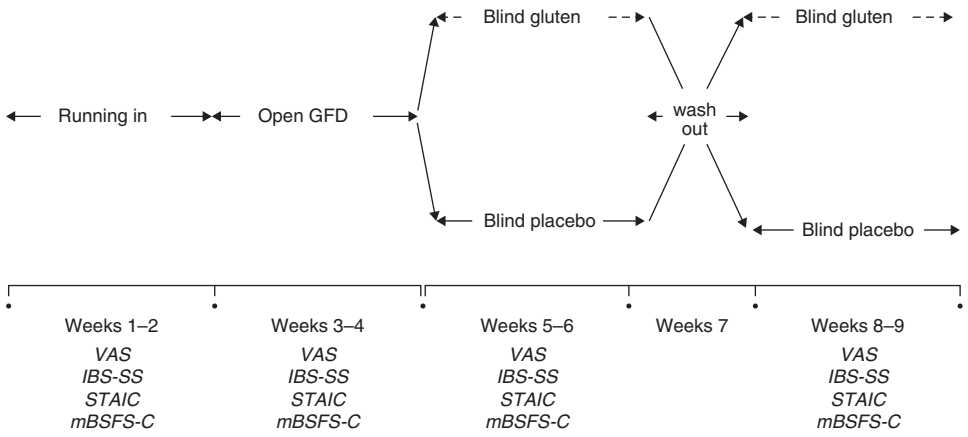

Figure 1. Crossover design of the study and the timing of clinical evaluations.

combined with the Faces Pain Scale on a daily basis to facilitate children understanding. The 0–10 mm VAS scale (0 no pain, 10 worst possible pain) included a horizontal color gradient (green–red), while the Faces Pain Scale comprises six faces ranging from a relaxed face to a face showing intense pain (15–17). VAS was used to grade all gastrointestinal and/or extra-intestinal symptoms each child reporting his own. We used a mBSFS-C (18–19) to monitor changes in intestinal function on a weekly basis.

IBS symptoms were evaluated weekly using a standardized questionnaire (IBS–SS) that includes five questions about pain, distension, bowel dysfunction, and quality of life/global well-being. Mild, moderate, and severe cases were indicated by scores of 75–175, 175–300, and >300, respectively. Patients scoring below 75 in this range are considered healthy (20).

To assess child anxiety, we used the STAIC (21–23).

All serological, allergological, genetic, and histological methods are described in the **Supplementary Material** online.

Outcome measures

The primary outcome was to identify the prevalence of NCGS in children who were referred for chronic functional gastrointestinal symptoms with a DBPC gluten challenge with crossover. Secondary outcomes were to identify clinical and/or laboratory parameters at baseline that can be predictive for NCGS and to describe NCGS clinical profile.

We adopted two different outcome measures to define a positive response to the DBPC gluten challenge. The first is the application of the Salerno criteria (2) based on a symptomatic response characterized by a decrease of at least 30% of the global VAS from the gluten challenge to the placebo challenge, and the second outcome is that used by Di Sabatino *et al.* (5), according to which true NCGS patients are defined as patients having a delta global VAS score $[(VAS_{gluten}-VAS_{placebo})/VAS_{gluten}\times 100]$ higher than the mean delta global VAS score +2 s.d.

Statistical analysis

A per-protocol analysis was applied to the trial. Normally, distributed grouped data were expressed as the means and standard deviation (±s.d.) and compared using paired and unpaired *t*-tests. Non-parametric grouped data were expressed as the means (95% CI) and compared using the Mann–Whitney rank sum test (paired) or Wilcoxon´s signed rank test (unpaired). Proportionate data were compared using Fisher´s exact test or the χ^2-test as appropriate. Differences between groups were analyzed using the two-tailed Student's *t*-test for independent samples. To compare the means of more than two samples, one-way analysis of variance was used. *P* values <0.05 were regarded as significant. The relationship of several risk factors to outcome was evaluated using logistic regression. Assuming a 30% positivity to the challenge, we estimated that 30 patients would be required for the study to have 80% power and a two-sided 5% significance level. The statistical analysis was performed using SPSS 13.0 (Chicago, IL, USA). The study adhered to the Declaration of Helsinki, was approved by the institutional ethical committee (DG no. 703/2013), and was registered at ClinicalsTrials.gov (NCT02431585).

All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Patients

The flow of patients involved in the trial from assessment for eligibility through follow-up is shown in **Figure 2**. Overall, a total of 1,114 children were screened for the study (415 in Bari, 299 in Rovereto, 200 in Cava dei Tirreni, 120 in Milano, and 80 in Roma). Of the children, 1,078 (96.7%) did not present any correlation of symptoms with gluten ingestion and were not considered candidates for the diagnostic phase. The remaining 36 children entered the run-in phase, and five patients (13.9%), who presented an improvement of symptoms (global VAS<3), exited the study. Thirty-one children proceeded to the open GFD, and three (8.3%) who did not report any improvement, were excluded. Thus, 28 children (77.7%) entered the gluten challenge phase. The baseline demographic characteristics and clinical and biochemical parameters of the children are reported in **Table 1**; no differences were found among the children who exited or continued

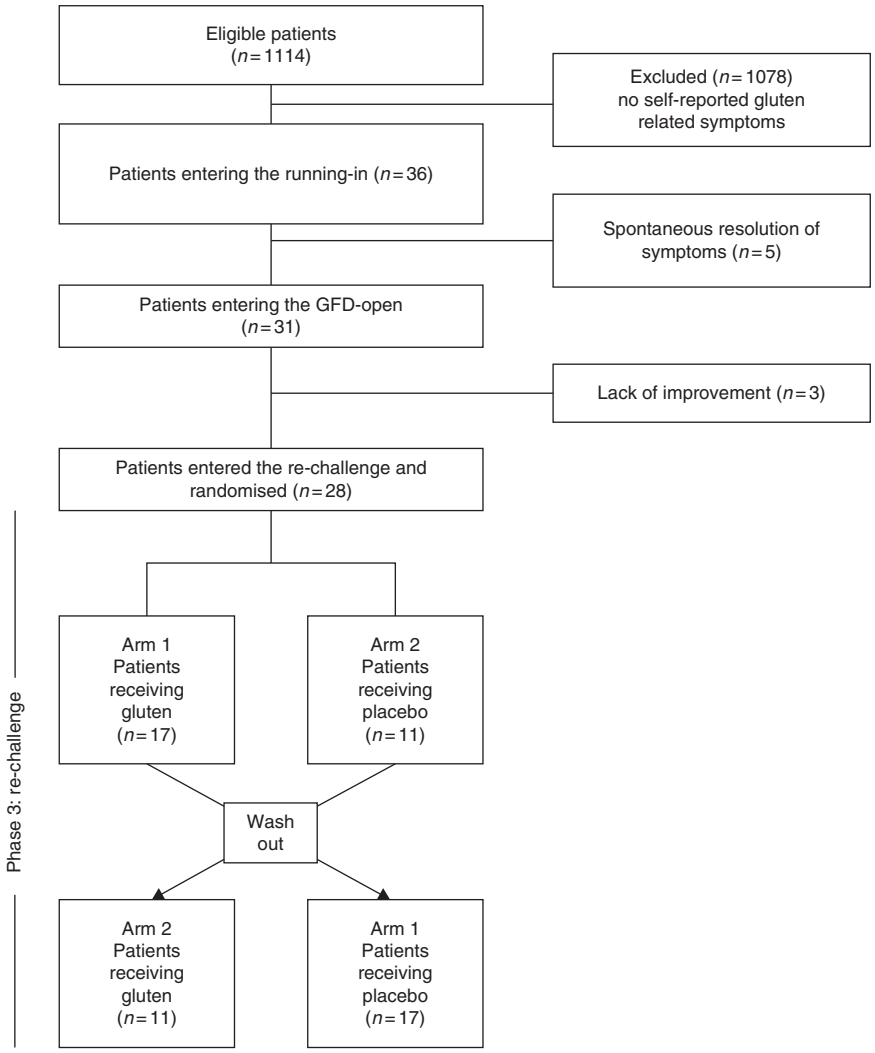

Figure 2. Flow diagram of patients into the trial from eligibility to the end of the challenge.

the diagnostic trial. The most common presenting features were fatigue (85%), abdominal pain (78%), headache (71%), and joint/muscle pain (57%).

Duodenal biopsy

Among 28 enrolled children, 15 (53%) carried the human leucocyte antigen predisposition and 6 (21%) who consented underwent endoscopy. In all, histology revealed the absence of proliferative and/or atrophic lesion with just a mild increase of intraepithelial lymphocytes in two (mean percentage of intraepithelial lymphocytes: 24.7 IEL/100).

Outcome of the gluten challenge

All 28 GFD-responsive children underwent the DBPC gluten challenge. According to the randomization, 17 children received sachets containing gluten in the first 2 weeks (W5–W6), followed by placebo for the second 2 weeks (W8–W9); 11 children received the opposite. Baseline characteristics were similar between the two groups. By dividing the randomized children on the basis of the result to the gluten challenge according to the Salerno criteria (2) (global VAS variation >30% between the gluten and the placebo challenge groups), 11 patients were found positive (39.2%; 95% CI: 23.6–53.6%), and 17 were found negative. The sequence of gluten/placebo allocation did not influence the result of the gluten challenge (p=NS). The demographic and basal clinical/biochemical parameters of the children who tested positive or negative in the gluten challenge are reported in **Table 1**. We registered no drop-out, and no sachets were returned after completion of the challenge and at least 80% of the content of each sachet was eaten.

All patients were available for final analysis, and none reported any side-effect during the trial.

Clinical scores

Overall, we found a decrease of all clinical scores (VAS, IBS–SS, STAIC) when patients started an open GFD (P<0.001 for

Table 1. Baseline demographic and clinical characteristics of the enrolled patients

Variable	GFD-responsive patients *n*=28	Re-challenge positive *n*=11	Re-challenge negative *n*=17	*P* value
Age (years)[a]	11.4±4.3	11.5±3.8	11.3±4.8	NS
Sex (male), *n (%)*	11 (39%)	4 (36%)	7 (41%)	NS
BMI (centiles)[a]	53±30.7	49±32.6	55±31.6	NS
Gastrointestinal				
Abdominal Pain	22 (78%)	10 (90%)	12 (70%)	NS
Diarrhea	11 (39%)	4 (36%)	7 (41%)	NS
Bloating	10 (35%)	5 (45%)	6 (35%)	NS
Dyspepsia	11 (39%)	3 (27%)	7 (41%)	NS
Mixed bowel habits	8 (28%)	3 (27%)	5 (29%)	NS
Nausea	4 (14%)	1 (9%)	3 (17%)	NS
Constipation	3 (10%)	0 (0%)	3 (17%)	NS
Regurgitation	1 (3%)	0 (0%)	1 (5%)	NS
Extra-intestinal[b]				
Fatigue	24 (85%)	11 (100%)	13 (76%)	NS
Headache	20 (71%)	9 (81%)	11 (64%)	NS
Joint/muscle pains	16 (57%)	6 (54%)	10 (58%)	NS
Skin rash	11 (39%)	4 (36%)	7 (41%)	NS
Irritability	2 (7%)	2 (18%)	0 (0%)	NS
Oral ulceration	2 (7%)	0 (0%)	2 (11%)	NS
Paresthesia	1 (3%)	1 (9%)	0 (0%)	NS
Inappetence	1 (3%)	0 (0%)	1 (5%)	NS
HLA-DQ2/8 pos[b]	15 (53%)	8 (72%)	7 (41%)	NS
Anti-gliadin IgG pos (>10 UA/ml)[b]	11 (39%)	4 (36%)	7 (41%)	NS
Hemoglobin (>12 g/dl)[a]	12.4±0.9	12.7±1	12.5±1.4	NS
Iron (>30 ng/ml)[a]	53.1±19	55±23.2	51±25	NS
Ferritin (>15 ng/ml)[a]	28.4±6.4	28.8±6.8	28.0±7.8	NS
AST (<30 U/ml)[a]	21.6± 4.9	22.8±3.8	21.0±5.8	NS
erythrocyte sedimentation rate (<20 mm/h)[a]	7.7±3.6	7.3± 2.9	7.6±2.6	NS

AST, aspartate aminotransferase; GFD, gluten-free diet; IgG, immunoglobulin G; HLA, human leucocyte antigen.
[a]Mean±s.d.
[b]*n* (%)

each score) (**Table 2**). No difference was found in the severity of the global VAS score during the treatment with gluten in comparison with the treatment with placebo during the gluten challenge (3.9±2.9 vs. 3.5±2.8; *p*=NS). The modifications of the clinical scores in the different phases of the study according to the outcome of the gluten challenge are reported in **Table 3** and presented in **Figure 3a-c**. There was no difference in the mean overall score between sequences of gluten/placebo allocation or the first/second period of the challenge.

According to Di Sabatino *et al* (5), we plotted the global VAS while children were on gluten (*X*-axis) vs. while they were on placebo (Y-axis) (**Figure 4**); when we did this, we observed that most children (11 of 28; 39%) clustered in a square area that was defined by an overall VAS of 5 (half of the maximal value) while ingesting both gluten and placebo, and 8 children were close to the dashed diagonal line (an equal degree of overall symptoms while ingesting either gluten or placebo). Four children (14%) were localized in the lower right region of the diagram (high positive delta between gluten and placebo); when we plotted these children on the basis of their delta global VAS scores and applied the definition of true NCGS (5), we found that only four children had an overall delta score greater the mean+2 s.d. (99.8±218). All were clustered in the lower-right region of the XY-diagram (grey ellipse). We have noticed that four patients (white circle) had a large increase in symptoms with placebo that however cannot be attributed to rice starch because the rice was allowed throughout all the study. This strengthens the need of a DBPC gluten challenge for a correct diagnosis.

Symptoms, diet, and laboratory data

We found no difference in the prevalence of presenting symptoms and/or the appearance of passed stools (as assessed by the mBSFS-C) and/or laboratory parameters among the children according to the result of the gluten challenge (**Table 1**). Of particular interest are the observations that some symptoms (fatigue, abdominal pain, headache, and joint/muscle pain) were correlated with gluten ingestion (**Supplementary Table**) and that NCGS patients experience a worsening of fatigue while on gluten (open and blind) ($P<0.001$; **Supplementary Figure**). No differences in the composition of the diet and in the amount of FODMAP consumption were detected during the different phases of the trial.

Prevalence of NCGS

The prevalence of NCGS in children with symptoms that are responsive to an open trial of GFD varies depending on the criteria used: according to the Salerno criteria (2), this prevalence is 39.2% (95% CI: 23.6–53.6%), but according to the criteria proposed by Di Sabatino *et al.* (5), the prevalence is 14.3% (95% CI: 5.7–31.5%). Considering the overall population of children who were referred for GI symptoms. we calculate that the prevalence of NCGS in this population varies from 0.36% (95% CI: 0.14–0.92%) (5) to 0.98% (95% CI: 0.5–1.8%) (2). This figure might be underestimated as we may not exclude that gluten might be responsible of symptoms that patients are unable to associate with its consumption.

DISCUSSION

The present study, the first pediatric study to use the DBPC gluten challenge with a crossover design, supports the existence of NCGS in childhood with FGIDs, and establishes the need to use this approach to finalize a diagnosis of NCGS, considering that

Table 2. Clinical scores in the overall population of children who completed the study (*n*=28)

Scores mean±s.d. (CI 95%)	phase 1 Run-in	phase 2 GFD open	phase 3 Gluten challenge gluten	phase 3 Gluten challenge placebo	*P* value (all times)
Global VAS	6.1±1.7 (5.4–6.8)	2.7±1.9 (2–3.5)	3.9±2.8 (2.8–5)[a]	3.5±2.7 (2.4–4.5)[a]	*P*<0.001
IBS–SS	313.8±105.3 (273–354.6)	113±62.2 (89–137.2)	162.2±107.7 (120.5–204)[a]	164.2±112.5 (120.6–207.9)[a]	*P*<0.001
STAIC	14.2±3.3 (12.9–15.4)	10.9±2 (10.1–11.8)	11.5±2.6 (10.4–12.5)[a]	11.3±2.3 (10.4–12.2)[a]	*P*<0.001

GFD, gluten-free diet; IBS–SS, irritable bowel syndrome–severity score; STAIC, State-Trait Anxiety Inventory for Children; VAS, visual analogue scale.
[a]=NS

Table 3. Modification of the clinical scores in the different phases according to the challenge result

Scores	Gluten challenge positive *n*=11	Gluten challenge negative *n*=17	*P value*
VAS mean±s.d. (CI 95%)			
Phase 1–Run-in	6.5±1.6 (5.3–7.6)	5.9±1.9 (4.9–6.8)	NS
Phase 2–Diagnostic GFD	2.9±1.3 (2–3.8)	2.6±2.2 (1.4–3.8)	NS
Phase 3–Rechallenge Gluten	5.3±2.2 (3.8–6.9)	3±.2.8 (1.6–4.5)	<0.005
Phase 3–Rechallenge Placebo	1±1.1 (0.3–1.9)	5±2.3 (3.8–6.2)	<0.001
IBS–SS mean±s.d. (CI 95%)			
Phase 1–Run-in	312.8±94.5 (49.2–376.3)	314.4±114.5 (255.5–373.4)	NS
Phase 2–Diagnostic GFD	92.6±21.6 (78–107.1)	126.3±75.8 (87.3–165.3)	NS
Phase 3–Rechallenge Gluten	206.3±102.9 (137.2–275.4)	133.8±103.9 (80.4–187.2)	<0.001
Phase 3–Rechallenge Placebo	73.3±25 (56.4–90)	223±107.8 (167.6–278.5)	<0.001
STAIC mean±s.d. (CI 95%)			
Phase 1–Run-in	14.8±3.5 (12.5–17.2)	13.7±3.2 (12–15.4)	NS
Phase 2–Diagnostic GFD	10.8±2.3 (9.3–12.4)	11±1.9 (10–12)	NS
Phase 3–Rechallenge Gluten	11.9±3.5 (9.5–14.2)	11.2±1.9 (10.2–12.2)	NS
Phase 3–Rechallenge Placebo	10.1±1.5 (9–11.2)	12±2.4 (10.8–13.3)	<0.005

GFD, gluten-free diet; IBS–SS, irritable bowel syndrome–severity score; STAIC, State-Trait Anxiety Inventory for Children; VAS, visual analogue scale.

at least one in every three diagnosis is disavowed. The present study initially planned to enroll only IBS patients, was immediately extended to the overall population of children with FGIDs because of an unexpected higher referrals of patients referring gluten associated symptoms not classified as IBS.

NCGS is characterized by intestinal and/or extra-intestinal symptoms that are related to the ingestion of gluten in subjects that are not affected by either CD or WA (1). The prevalence of this condition is unknown and, according to the largest series of self-reported gluten avoidance studies, ranges from 0.5 to 13% of the general population (24).

We were the first to describe a pediatric series of NCGS patients using an open challenge (7); however, we became aware that some of these diagnoses might have been influenced by the nocebo effect. Therefore, according to the most recent recommendations(2), we planned the first DBPC pediatric gluten challenge. Currently, in people's imagination, gluten is responsible for a series of symptoms that encompass both intestinal and extra-intestinal symptoms that can alter the quality of life. This opens the discussion of whether gluten might be able to cause symptoms in the absence of CD or WA. Products of gluten digestion can lead to the up-regulation of pro-inflammatory cytokines, epithelial cell dysfunction, increased IL-15 production, enterocyte apoptosis (25) and abnormal smooth muscle contractility (mouse model) (26). The biological plausibility of gluten toxicity needs to be balanced with the wide perception of the nocebo effect, which, according to recent data, might be as high as 40% (27). On this basis, the self-prescription of gluten restriction should be strongly discouraged for several reasons of both clinical and social interest, such as the possible preclusion of a proper diagnosis of CD, the risks associated with an exclusion diet, and the unjustified economic burden (28).

DBPC gluten challenge studies with a crossover design in patients with suspected NCGS are accumulating for adults and show conflicting results. In a retrospective series of 920 patients with FGIDs, Carroccio *et al.* (3) demonstrated that ~30% were

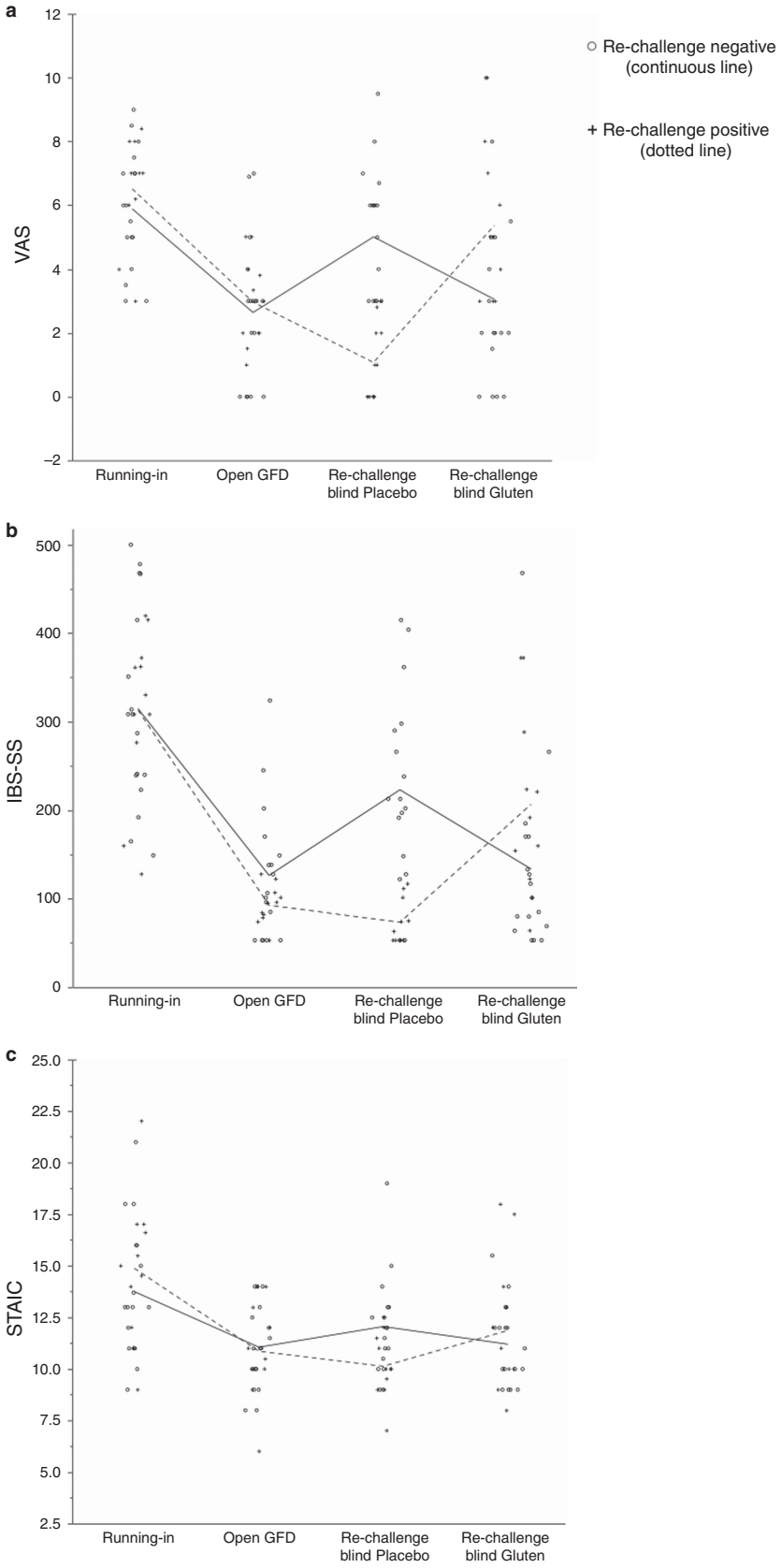

Figure 3. Comparison of clinical scores in the different phases of the study according to the outcome of the gluten challenge.

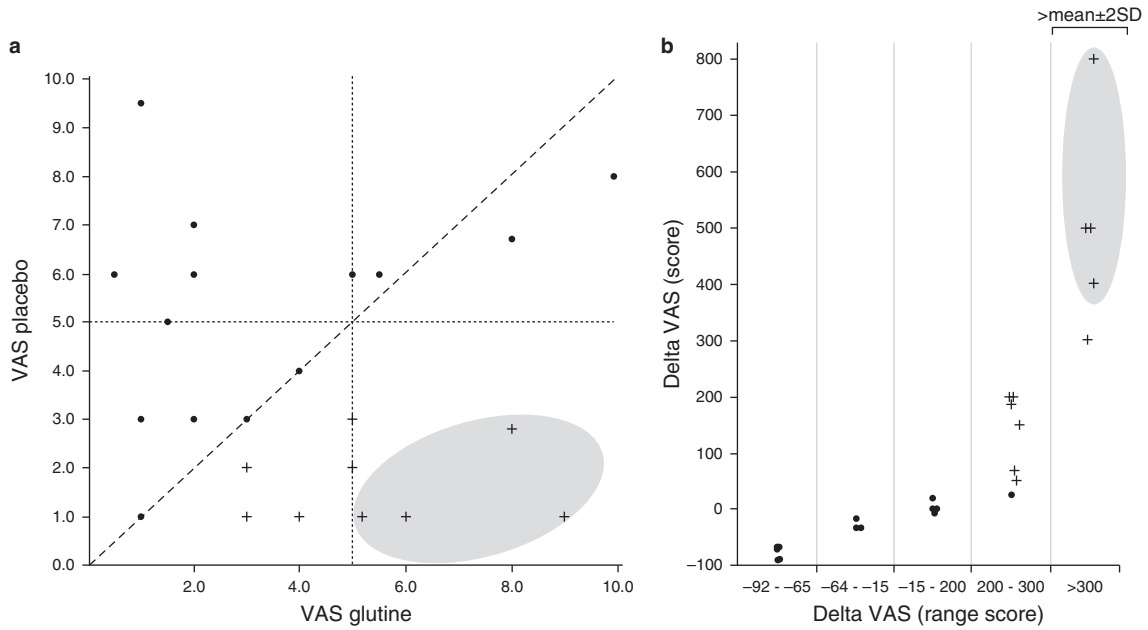

Figure 4. Distribution of patients according to their VAS while on gluten and placebo; patients on the diagonal line have an equal response to gluten and placebo, children in the lower left quadrant experience a mild degree of overall response (<5) to either gluten or placebo (panel **a**). Panel **b** shows the distribution of patients according to their delta global VAS scores; according to our definition of true NCGS patients, four children had an overall delta score greater than the mean+2SD (99.8±218). All four clustered in the lower-right region of the diagram shown in panel **a** (gray ellipse).

positive when challenged. In a group of 37 IBS subjects with self-reported NCGS, Biesiekierski *et al.* (29) showed no evidence of a specific effect of gluten in patients placed on a low FODMAP diet with a low prevalence of NCGS (8%). In 35 patients with self-reported NCGS, Zanini *et al.* (4) found a prevalence of NCGS of 33%, whereas Di Sabatino *et al.* (5), using a strict diagnostic definition, reached the diagnosis in 3 out of 59 patients (5%). Most recently, in 98 adults with IBS responsive to a GFD, Elli *et al.* (6) showed a positive response to the challenge in 28% of the cases. Although methodological flaws related to the heterogeneity of the population under investigation, outcome measures and challenge design (type of protein, dose of gluten, delivery mode, and duration of wash-out period), all trials clearly demonstrated that the overwhelming majority of patients who present gluten-related symptoms are not in fact affected by NCGS. Our experience in children strengthens these conclusions as >60% of the suspected diagnoses were refuted by a gluten challenge, which should be considered mandatory for the diagnosis until a reliable marker of NCGS becomes available (27).

One of the main questions arising from our work is how to determine whether a gluten challenge should be considered positive. Indeed, we found a profound difference in positivity when applying the Salerno Criteria (2) or the approach suggested by Di Sabatino *et al.* (5). We believe that these two results are not conflicting and simply provide upper and lower figures of prevalence of this condition. Indeed, all four children identified by the Di Sabatino approach were also identified by the Salerno criteria and represent the worst cases. We believe that a possible way to integrate the two approaches would be to increase the cutoff of the symptomatic response to a point higher than 30% of the baseline score (2); however, the calculation of this estimate was far beyond the aim of our study.

At present, there is no agreement among studies regarding the dose of gluten to administer; in the absence of data or suggested doses for children, we decided to give a dose of 10 grams of gluten considering that a slightly high dose of gluten might be more accurate for a diagnostic challenge without affecting tolerability. We administered the gluten as a powder to be dissolved in food in two or three portions per day to mimic the daily ingestion of gluten, to allow masking, and to avoid any non-specific effect on gastrointestinal motility and fermentation (30). We chose a 2-week duration for the gluten challenge to allow the identification of children with mild or fluctuating symptoms and selected a wash-out period of 1 week as gluten avoidance is followed by rapid symptom resolution. We are confident of the validity of this approach since the level of compliance was high (highly motivated patients) with no drop out.

We believe that our study has several strengths. This is the first pediatric DBPC challenge study, which is considered the gold standard, to demonstrate that gluten might be responsible for symptoms in children and that this approach is mandatory for the identification of cases. We showed that NGCS should be suspected mainly in those children presenting severe extra-intestinal symptoms and that, as for adults (1), no predictive laboratory tests can help in identifying cases. Finally, we estimate that among those children who were presented to a tertiary referral center for gluten-related gastrointestinal symptoms, at least one in seven (but up to one in three) warrant a gluten elimination

diet; this figure is not alarming if we consider that in our experience, we expect 1 out of every 280 children (1 in 100 using the Salerno criteria) who are referred for chronic GI symptoms to have NCGS.

We are aware of some limits of our study. We were unable to identify a serological marker for this condition, and we had to use the adult experience to build a model of pediatric challenge using a gluten dose and mode of administration that were adapted for use with children. We can not answer the question whether is gluten or wheat to be responsible for symptoms; indeed, the marked reduction of all the scores during the open GFD period, apart from the well-known nocebo, effect might be secondary to the wheat exclusion that could not be demonstrated by the gluten challenge (31–33). We acknowledge that the study is underpowered according to the estimated sample size that required 30 patients while we enrolled only 28 for the final phase of the study. Moreover the study population may not be representative to the overall NCGS population mainly for the self-reported association of symptoms with gluten ingestion, which may infer the results. The imbalance between the two arms might could also expose to a bias, however, we did not have a crossover effect as shown by the fact that positivity to the challenge was prevalent (although not significant) in the sequence gluten-placebo, and the global VAS scores did not differ among the sequences. Finally, the prevalence of NCGS might have been underestimated as only children with gluten self-reported symptoms, entered the challenge phase.

In conclusion, we learned from this trial that we are at the beginning of our understanding of this condition, which does not spare children but might be currently overestimated. We conclude by quoting the philosopher Immanuel Kant, "all our knowledge begins with the senses, proceeds then to understanding, and ends with reason". NCGS begins in the gut feeling of patients, and we are still in the process of understanding it, hoping that reason is not too far behind.

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CONFLICT OF INTEREST

Guarantor of the article: Ruggiero Francavilla is accepting full responsibility for the conduct of the study. He has access to the data and control of the decision to publish.

Specific author contributions: R.F., F.C., A.G., and F.I. designed, conceived, organized, and coordinated the study; R.F., F.C., A.G., L.V., S.C., C.P., V.G., E.V., E.D., and F.I. carried out the medical clinical work; S.D. carried out the dietetic clinical work, R.F. and F.C. drafted the manuscript; and R.F. carried out the statistical analysis. All authors have read and revised the manuscript. All authors approved the submission of this version of the manuscript and take full responsibility for the manuscript. Writing Assistance: American Journal Experts (USA).

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Study highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ NCGS is characterized by intestinal and/or extra-intestinal symptoms that are related to the ingestion of gluten in subjects that are not affected by either CD or WA.
- ✓ NCGS is receiving widespread interest from the general public, and many patients often start a self-prescribed gluten-free diet without any medical indications.
- ✓ Many studies have been performed on NCGS in adults; however, few data are available in children.

WHAT IS NEW HERE

- ✓ In children, NCGS does exist however is less common compared with adults.
- ✓ DBPC gluten challenge is mandatory for the diagnosis as the diagnosis is ruled out in >60% of the self reported cases.

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