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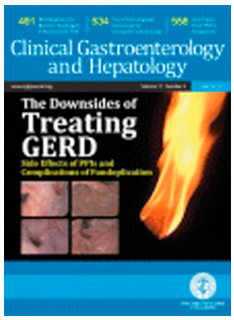

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Small Amounts of Gluten in Subjects with Suspected Nonceliac Gluten Sensitivity: a Randomized, Double-Blind, Placebo-Controlled, Cross-Over Trial

Short title: Small gluten amounts and nonceliac gluten sensitivity

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Abbreviations: AGA, anti-gliadin antibodies; FODMAP, fermentable, oligo-, di- and monosaccharides and polyols; GFD, gluten-free diet; NCGS, nonceliac gluten sensitivity.

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Abstract

Background & Aims: There is debate over the existence of nonceliac gluten sensitivity (NCGS) -intestinal and extra-intestinal symptoms in response to ingestion of gluten-containing foods by people without celiac disease or wheat allergy. We performed a randomized, double-blind, placebo-controlled, cross-over trial to determine the effects of administration of low doses of gluten to subjects with suspected NCGS.

Methods: We enrolled 61 adults without celiac disease or wheat allergy who believe ingestion of gluten-containing food to be the cause of their intestinal and extra-intestinal symptoms. Participants were randomly assigned to groups given either 4.375 g/day gluten or rice starch (placebo) for 1 week, each via gastro-soluble capsules. After a 1 week of gluten-free diet, participants crossed over to the other group. The primary outcome was the change in overall (intestinal and extra-intestinal) symptoms, determined by established scoring systems, between gluten and placebo intake. A secondary outcome was the change in individual symptom scores between gluten vs placebo.

Results: According to the per-protocol analysis of data from the 59 patients who completed the trial, intake of gluten significantly increased overall symptoms compared with placebo (P=.034). Abdominal bloating (*P*=.040) and pain (*P*=.047), among the intestinal symptoms, and foggy mind (*P*=.019), depression (*P*=.020), and aphthous stomatitis (*P*=.025), among the extra-intestinal symptoms, were significantly more severe when subjects received gluten than placebo.

Conclusions: In a cross-over trial of subjects with suspected NCGS, the severity of overall symptoms increased significantly during 1 week of intake of small amounts of gluten, compared with placebo. Clinical trial no: ISRCTN72857280.

KEY WORDS: extraintestinal; gluten; intestinal; nonceliac gluten sensitivity; placebo

Background and Aims

Gluten sensitivity was defined by an international panel as the occurrence of intestinal and extraintestinal symptoms related to the ingestion of gluten-containing food in subjects without wheat allergy or celiac disease.[1] Nonceliac gluten sensitivity (NCGS) is now considered a more proper term to distinguish this condition from celiac disease.[2,3]

NCGS has raised considerable interest and debate in both the medical and non-medical literature. Public awareness of NCGS is higher than that of celiac disease,[4] and crowded online forums guided patients to eventually reach their own self-diagnosis.[5] Nevertheless, the very existence of NCGS as a discrete entity was questioned,[6,7] and in the absence of intestinal lesions, specific antibodies or any other biomarker, the absolute need for an optimal diagnostic algorithm and shared diagnostic criteria was reiterated.[2] Most of the information on NCGS, such as its high prevalence,[1] the activation of the innate immunity as a preferential pathogenic mechanism,[8,9] the existence of a specific mucosal cytokine profile,[9,10] and its clinical spectrum[11-13], was obtained from patients who are mainly self-reported to be gluten-sensitive. Since at present nobody knows how many of these patients are really affected by NCGS, we carried out a double-blind, placebo-controlled, cross-over gluten challenge trial on patients suspected of having NCGS.

Patients & Methods

Study design and population.

The study was a prospective, randomized, placebo-controlled, cross-over trial that compared the effects of a daily dose of 4.375 grams of gluten with placebo in patients strongly suspected of having NCGS. The patients and the physicians who administered the interventions were blinded. Enrollment started in September 2012, and follow-up was

completed in January 2014. The Ethics Committee at both clinical sites (Pavia and Bologna) approved the study (trial registration number: ISRCTN72857280; http://www.controlled-trials.com/ISRCTN72857280), and patients provided written informed consent. All authors had access to the study data and have reviewed and approved the final manuscript.

One-hundred-eighteen adult patients were consecutively referred between October 2012 and November 2013 to two Italian Celiac Centers (Pavia and Bologna) because of persistence of relevant intestinal and extraintestinal symptoms believed by themselves to be caused by food containing even low doses of gluten, such as a sandwich or two slices of white bread, and affecting their quality of life. Only 92 patients -all under gluten-containing diet at the time of screening for at least two months- underwent *ad hoc* screening (Figure 1) (See Supplementary Methods). Among them, 61 patients (39 enrolled in Pavia and 22 in Bologna) were randomized in the trial (Table 1).

Randomization and interventions.

Patients were randomized according to a computer-generated list of random numbers held by an independent observer to either the gluten or the placebo treatment group (Figure 2). Patients were asked to fill in a daily questionnaire in order to assess a rating scale of both intestinal and extraintestinal symptoms (see Supplementary Methods) over a 5-week period. Participants were asked to follow a strict gluten-free diet (GFD) starting one week before the randomization (W_0) and continuing until the end of the study period (W_5). Compliance with the GFD was assessed by using a validated questionnaire.[14] At W_1, patients were given either gastrosoluble capsules containing purified wheat gluten (10 capsules ingested on no more than two occasions over the day, corresponding to a daily gluten intake of 4.375 grams, equivalent to ~2 slices of white bread) or gastrosoluble capsules containing rice starch (10 capsules corresponding to a

daily rice starch intake of 4.375 grams) as placebo for one week. Rice starch was chosen as the placebo because it is the most readily absorbable of the complex carbohydrates, and thus less fermentable, in the intestinal tract.[15] At the end of the first treatment week, patients from both arms continued only their wash-out from gluten, without taking any capsule. Subsequently, at $W_{3,}$ individuals belonging to the first arm were given placebo capsules, while individuals belonging to the second arm were given gluten capsules. After the second treatment week, all patients continued with their wash-out from gluten. Capsules were kindly provided by Giuliani Pharma (Milan, Italy).

Outcomes.

The primary outcome was the change in the weekly overall symptom score, as assessed by the sum of intestinal and extraintestinal scores, between the 1-week treatment with gluten and the 1-week treatment with placebo. Patients were asked to grade daily 15 intestinal symptoms and 13 extraintestinal symptoms (See Supplementary Methods).[16] Secondary outcomes were (*i*) the change in individual symptom scores between 1-week treatment with gluten and 1-week treatment with placebo, (*ii*) the identification of patients with true NCGS, and (*iii*) to verify whether laboratory parameters at baseline might be predictive of true NCGS. True NCGS patients were defined as having at the end of the trial a *delta* overall score -calculated by subtracting the weekly overall score under placebo from that under gluten- higher than the mean *delta* overall score +2SD. Laboratory parameters included serum IgG AGA, fecal calprotectin, HLA genotyping and intraepithelial lymphocyte density (See Supplementary Methods).

Statistical analysis.

A *per protocol* approach was applied for this cross-over trial, and the statistical analysis was conducted on patients with available data in both study periods (two patients

were excluded due to missing data, see below). The determination of the sample size was made by using a two-side testing framework with an α error of 0.05 and a β error of 0.2, as the prospective primary hypothesis was that the 1-week treatment with gluten would result in a different severity of the overall score, as assessed by the sum of intestinal and extraintestinal scores, than would the 1-week treatment with placebo. Assuming a within-patient comparison and a SD of the overall score of 40, we estimated that 58 patients would be needed in order to achieve a power of 80%, at a 2-sided 5% significance level, if the true difference was 15. An analysis of variance (ANOVA) for cross-over design was conducted (See Supplementary Methods).

Results

According to the cross-over trial design, 31 patients (Arm1) started with gluten capsules in the first treatment week (W_1-W_2), followed by placebo capsules in the second one (W_3-W_4), while 30 (Arm2) started with placebo capsules in the first treatment week, followed by gluten capsules in the second one (Figure 2). Baseline characteristics were similar between the groups (Table 1). Fifty-nine of the 61 randomized patients completed the 5-week trial as *per protocol*, since two patients discontinued the study prematurely because of intolerable symptoms (Figure 1): one patient withdrew after two days of the first treatment week (Arm2, placebo) because of abdominal pain and nausea, while the other withdrew after two days of the first treatment week (Arm1, gluten) because of abdominal pain and diarrhea.

Primary Outcome.

According to the *per protocol* analysis on the 59 patients who completed the trial, the 1-week treatment with gluten resulted in a significantly (p=0.034) higher severity of

the overall score in comparison to the 1-week treatment with placebo (Table 2). The median overall score after 1-week of gluten consumption was 48 (range 1-156), while after 1-week of placebo it was 34 (range 0-178). However, when we applied the ANOVA analysis of variance for cross-over design, the overall score of the 59 patients in the first week period (W_1-W_2) (median 50, range 2-178) was significantly (p=0.009) higher in comparison to that observed in the second one (W_3-W_4) (median 33, range 0-155) (Table 2). No significant difference (p=0.242) was found in the overall score between sequences, i.e. (gluten→placebo) and (placebo→gluten). The daily overall score over the gluten/placebo treatment week is shown in Figure 4 (panel A). At Day0 no significant difference was found in the mean overall score between the two subgroups of patients who had experienced or not a self-prescribed GFD.

Secondary Outcome.

When we plotted the weekly overall score under gluten (X axis) and that under placebo (Y axis) in an XY-diagram for each subject, we observed that most of the patients (44 of 59; 74%) clustered in a squared area defined by an overall score < 90, both under gluten and under placebo (Figure 3A). Among the 44 patients contained in the squared area, 31 -those in the pink hexagonal area- were very close to the dashed diagonal line, i.e. they complained to an equal degree of overall symptoms either under gluten or placebo. Our attention was conversely focused on the 9 patients (15%) localized in the lower right region of the diagram, that is on those patients strongly suspected to be true gluten-sensitive according to their high positive gap between gluten and placebo scores. Thus, we plotted patients on the basis of their *delta* overall scores, which ranged from -103 to +156, with a mean (SD) value of 12.2 (50.4) (Figure 3B, dashed line). According to our definition (mean +2SD), the cut-off level of the *delta* overall score was +113 (dotted line). Only three patients had a *delta* overall score > 113, and thus were identified as true

gluten-sensitive. Of note, these three patients (Figure 3B, green ellipse) were three of the four patients who clustered in the lower-right region of the XY-diagram in Figure 3A (green ellipse). We also analysed the intestinal score (Supplementary Figure 1) and the extraintestinal score (Supplementary Figure 2) separately, and we obtained results comparable to those derived from the analysis of the overall score. In particular, 8 patients (13%) had a worsening of intestinal symptoms under gluten as they were localized in the lower right region of the diagram (Supplementary Figure 1B), and 6 (10%) had a worsening of extraintestinal symptoms under gluten (Supplementary Figure 2B).

A further secondary endpoint of the study was to analyze individual symptoms. As regards intestinal symptoms, three among the 15 investigated, i.e. abdominal bloating ($p=0.040$) and abdominal pain ($p=0.047$), were significantly worsened by gluten in comparison to placebo (Figure 4, panels B-C). Abdominal pain and bloating were the most scored intestinal symptoms over the week under gluten treatment, followed by wind (Supplementary Table 1). As regards extraintestinal symptoms, three among the 13 investigated, i.e. foggy mind ($p=0.019$), depression ($p=0.020$) and aphthous stomatitis ($p=0.025$), were significantly worsened by gluten in comparison to placebo (Figure 4, panels D-E). Among the three extraintestinal symptoms who were significantly worsened by gluten, only foggy mind was among the first most scored extraintestinal symptoms over the gluten week (Supplementary Table 2). When we assessed the percentages of patients complaining of individual symptoms under gluten treatment, we observed that among the five most prevalent symptoms, three were intestinal (abdominal pain, bloating and wind), and two were extraintestinal (headache and tiredness) (Supplementary Table 3). A significant positive correlation was found between symptom prevalence and overall score under gluten both for intestinal ($r_S=0.92$, $p<0.0001$) and extraintestinal symptoms ($r_S=0.90$, $p<0.0001$). None of the intestinal or extraintestinal symptoms showed higher mean scores during the placebo compared with gluten.

Finally, we analyzed laboratory parameters, such as serum IgG AGA, fecal calprotectin, intraepithelial lymphocyte density and HLA genotyping, all assessed at baseline and under gluten-containing diet (see Supplementary text).

Discussion

Gluten proteins are among the most complex protein systems due to numerous components of various sizes, and due to further variability determined by genotype, growing conditions and technological processes.[17] The complexity of the gluten structure couples with the multifaceted pathological and clinical spectrum of gluten-related conditions which, in addition to celiac disease,[18] includes wheat allergy and a number of extraintestinal disorders.[19] Moreover, gluten administration for long periods or in high doses can cause symptoms and intestinal lesions in unaffected relatives of celiac patients and in nonceliac individuals with altered immunity.[20]

NCGS, a possible further example of this complexity, has been attracting increasing attention in recent years. A growing number of patients claim that gluten is responsible for both intestinal and extraintestinal symptoms to an extent that impairs their quality of life. On this basis, self-prescription of gluten withdrawal is becoming increasingly common, but this behaviour should be strongly discouraged as it may lead to the consequent preclusion of a proper diagnosis of celiac disease and to a high and unjustified economic burden.[2] Since a reliable marker of NCGS is not readily available at present, double-blind, placebo-controlled trials are mandatory to ascertain this condition.

Unlike previous prospective double-blind, placebo-controlled gluten challenge trials,[21-23] the topic of this study is not the patients referred for irritable bowel syndrome based on Rome III criteria, but those referred to tertiary centers due to intestinal and extraintestinal symptoms caused by the ingestion of even low doses of gluten. In order to

overcome the most common biases linked to double-blind, placebo-controlled trials,[24,25] we here adopted a number of anticipatory measures, including the pre-testing demonstration that gluten capsules were indistinguishable from placebo capsules in taste and appearance, the exclusion of patients reporting minimal symptoms, and the use as the primary endpoint of change in the global response, which is what patients want definitely to improve. Actually, specific changes in bowel habits or single extraintestinal symptoms might not reflect the complexity of such a polymorphic syndrome. On the other hand, as global measures may miss some specific effects, we also analyzed the influence of gluten on single intestinal and extraintestinal symptoms.

Further guarantors of the validity of this study are the good level of patient compliance to both treatments and GFD, carefully verified throughout the trial, the lack of carry-over effects demonstrated by the absence of a significant difference in the mean overall score between sequences, and the low rate of patient withdrawal. Nevertheless, we observed an order effect, demonstrated by the significant difference in the mean overall score between periods (period 1 over period 2), which was present regardless of the nature of the treatment. This effect, which is unavoidable in cross-over trials,[22,26,27] is a well known consequence of the psychological impact of entering into a trial.[25]

In the present study, gluten was tested in a daily dose considerably lower than that administered in previous double-blind, placebo-controlled trials (16-20 grams),[21,22,28] but certainly more physiologic (4.375 grams are roughly equivalent to one sandwich or two slices of wheat bread), and still able to induce either symptoms in NCGS patients[22] or small bowel lesions in celiac patients.[29] Moreover, it should be underlined that higher gluten doses may evoke in normal subjects non-specific effects on gastrointestinal fermentation[30] or motility (paper in preparation). Based on the observations of Biesiekierski et al.,[21] who found that symptoms appeared in the first seven days after starting gluten administration, we believed a 1-week exposure to gluten to be sufficient. In

addition, longer treatment periods are supposed to be burdened by higher non-adherence rates,[25] surprisingly not reported by other longer gluten challenge trials. A possible limitation of our study is the relatively short period of wash-out from gluten. However, the low mean overall score at Day0 for all the patients, and the lack of a significant difference in the mean overall score at Day0 between the two subgroups of patients who had experienced or not a self-prescribed GFD, indicates that 1-week wash-out from gluten was enough to neutralize gluten-dependent symptoms complained during the baseline gluten-containing diet.

Actually, we found that the overall symptom score was significantly higher under gluten in comparison to placebo. However, when we examined the individual patients' overall scores we found that only a minority of the participants experienced a real worsening of symptoms under gluten. While it is possible that the global evaluation of the symptoms may in some way have attenuated the effect played by gluten on predominant symptoms, i.e. abdominal bloating and pain, we do acknowledge that the relevance of NCGS should be reappraised. This view is also supported by the evidence that in the vast majority of patients the clinical weight of gluten-dependent symptoms is irrelevant in the light of the comparable degree of symptoms experienced with placebo. If we look at the distribution of *delta* overall scores (gluten minus placebo), it is not surprising to note that a fair number of patients are victims of the *nocebo* effect, which was extensively proved through double-blind, placebo-controlled trials.[31,32]

In keeping with previous studies,[21,22] we found that both abdominal pain and bloating were significantly worsened by gluten. Moreover, both these symptoms were the most scored over the gluten treatment week, and the most prevalent among all the participants in the trial. This is quite an interesting finding when considering the results of recent surveys conducted on large cohorts of patients merely suspected for NCGS, showing that abdominal pain and bloating are complained of by up to 80% of patients.[11-13]

Among the extraintestinal symptoms, foggy mind, depression and aphthous stomatitis were significantly worsened by gluten, although unlike intestinal symptoms they were not among the most scored symptoms during the 1-week treatment with gluten. The observation that short-term exposure to gluten induces depression is remarkable, and this result is supported by a recent double-blind, placebo-controlled, cross-over study in which depression was assessed by an *ad hoc* psychiatric score.[33] The direct highly significant correlation between symptom score and symptom prevalence at both intestinal and extraintestinal level is indirect proof of the validity of our findings.

We acknowledge that our study does not provide any progress in identifying possible biomarkers of NCGS -neither serum IgG AGA nor intraepithelial lymphocytes correlated with either the overall response to gluten or the *delta* overall score- and in clarifying the pathogenic mechanisms underlying NCGS. Experiments aimed at defining the cytokine milieu in the duodenal mucosa of the patients enrolled in this trial are being conducted in our laboratory, and preliminary data do not seem to support the involvement of either innate or adaptive immune mechanisms in this condition.

In conclusion, in the present trial most patients showed approximately equal degrees of overall symptoms under either gluten or placebo, although overall symptoms were significantly worsened by gluten in comparison to placebo. As regards the identification of the true gluten-sensitive patients, it should be cautiously interpreted due to the lack of a control group of non-gluten-sensitive subjects, and it does not represent a crucial evidence in favor of the existence of this new syndrome. We cannot exclude the possibility that these patients merely had increased visceral or extraintestinal hypersensitivity to gluten, nor that higher gluten doses could have selected a larger cohort of sensitive patients. A greater understanding of the gut/gluten relationship can probably be obtained by dissecting the mechanisms driven by the administration of gluten in healthy volunteers.

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Figure Legend

Figure 1. Study flow diagram. FODMAP, fermentable, oligo-, di- and monosaccharides, and polyols.

Figure 2. Cross-over design of the trial. Grey and green panels represent period 1 and period 2, respectively. W, weekly appointment.

Figure 3. **A.** Distribution of patients according to their weekly gluten and placebo overall scores. The closer patients are to the dashed diagonal line, the more comparable their degrees of response are to either gluten or placebo. The squared area incorporates patients experiencing mild degrees of overall response (<90) either with gluten or placebo (44 of 59). Most of them (31 of 44) had comparable overall scores either with gluten or placebo (pink hexagonal area). **B.** Distribution of patients according to their *delta* weekly overall score, calculated by subtracting the overall score under placebo from that under gluten. The mean (SD) *delta* weekly overall score was 12.2 (50.4) (dashed line). Only three patients had a *delta* overall score higher than the fixed cut-off (mean+2SD=113). These three patients (green ellipse) were three of the four patients who clustered in the lower-right region of the diagram in panel A (green ellipse).

Figure 4. Comparison of scores of overall symptoms in the gluten and placebo treated groups over the 1-week treatment (A), and comparison of scores of those intestinal (B,C) and extraintestinal symptoms (D-E) which were significantly worsened by gluten. Data shown represent the mean overall score (SEM) at each day.

Table 1. Baseline characteristics of the 61 randomized patients

Parameters	All patients n=61	Patients starting with gluten (Arm 1)* n=31	Patients starting with placebo (Arm 2)* n=30
Mean age (*years*)	39.3	35.9	42.4
Females (*n*)	53	27	26
Mean duration of symptoms (*months*)	14.3	14.7	13.8
Previous self-prescribed gluten-free diet (*n*)	51	27	24
Mean duration of previous gluten-free diet (*months*)	11.1	11.7	10.4
Previous diagnosis of food intolerance *(n)*	20	8	12
Previous diagnosis of allergy (*n*)	7	4	3
Previous upper endoscopy (*n*)	36	19	17
Previous lower endoscopy (*n*)	8	5	3
Familiarity for CD (*n*)	9	5	4
Mean BMI (kg/m^2)	22.4	21.9	22.5
Iron-deficiency anemia (*n*)	6	3	3
HLA-DQ2/DQ8-positive (*n*)	16	8	8
Serum AGA IgG-positive (*n*)	16	7	9
Fecal calprotectin-positive (*n*)	0	0	0
Mean IEL (*per 100 IEC*)	25.1	23.8	25.3

*See Appendix Figure 1 for trial design. AGA, anti-gliadin antibodies; BMI, body mass index; CD, celiac disease; HLA, human leukocyte antigen; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte.

Table 2. Mean and median values of the weekly overall symptom score by *treatment, period* and *sequence in the 59 patients who completed the study**

	N	Mean	SD	Min	Median	Max	*P*-value
Treatment							
Gluten	59	56.9	40.4	2	50	178	
Placebo	59	43.7	34.9	0	33	155	0.034
Period							
1 (W_1-W_2)	59	58.6	40.7	1	48	156	
2 (W_3-W_4)	59	42.0	36.1	0	34	178	0.009
Sequence							
Gluten→Placebo (Arm1)*	59	54.8	37.9	0	47	156	
Placebo→Gluten (Arm2)*	59	46.0	35.7	1	35	178	0.242

*See Figure 2 for trial design. N, number of patients; SD, standard deviation; W, weekly appointment.

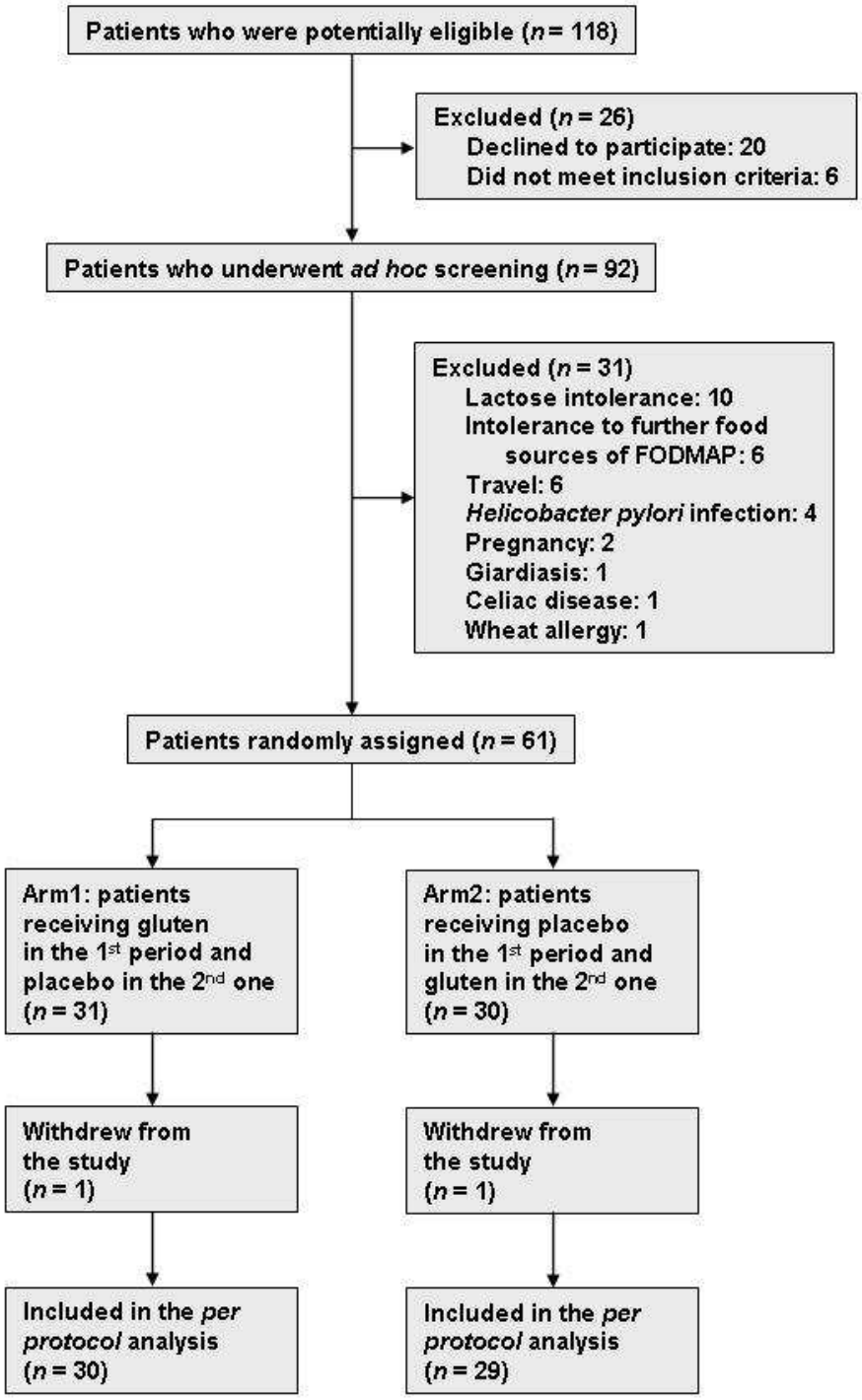

Patients who were potentially eligible (n = 118)
Excluded (n = 26)
Declined to participate: 20
Did not meet inclusion criteria: 6
Patients who underwent ad hoc screening (n = 92)
Excluded (n = 31)
Lactose intolerance: 10
Intolerance to further food sources of FODMAP: 6
Travel: 6
Helicobacter pylori infection: 4
Pregnancy: 2
Giardiasis: 1
Celiac disease: 1
Wheat allergy: 1
Patients randomly assigned (n = 61)
Arm1: patients receiving gluten in the 1st period and placebo in the 2nd one (n = 31)
Arm2: patients receiving placebo in the 1st period and gluten in the 2nd one (n = 30)
Withdrew from the study (n = 1)
Withdrew from the study (n = 1)
Included in the per protocol analysis (n = 30)
Included in the per protocol analysis (n = 29)

Figure 1

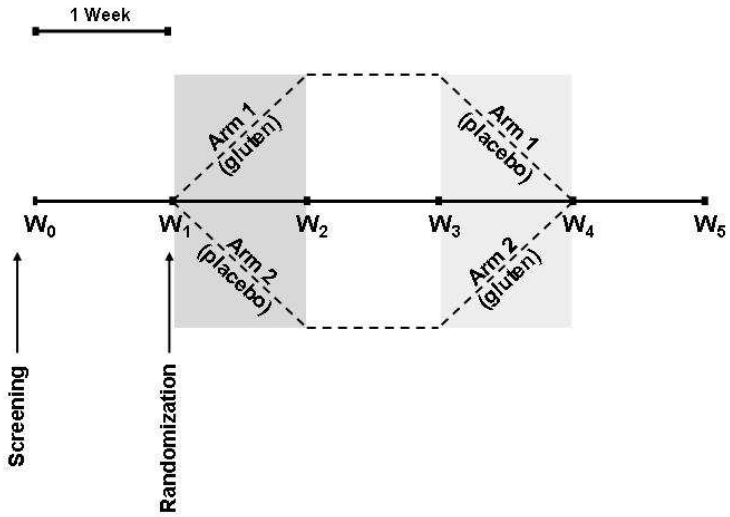


Figure 2

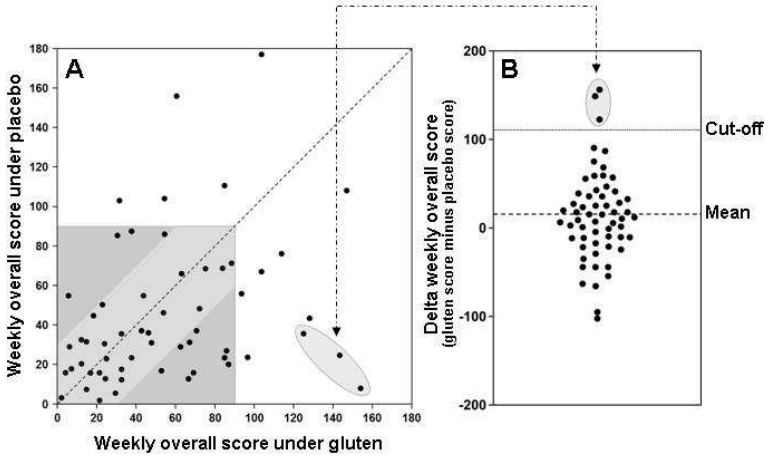


Figure 3

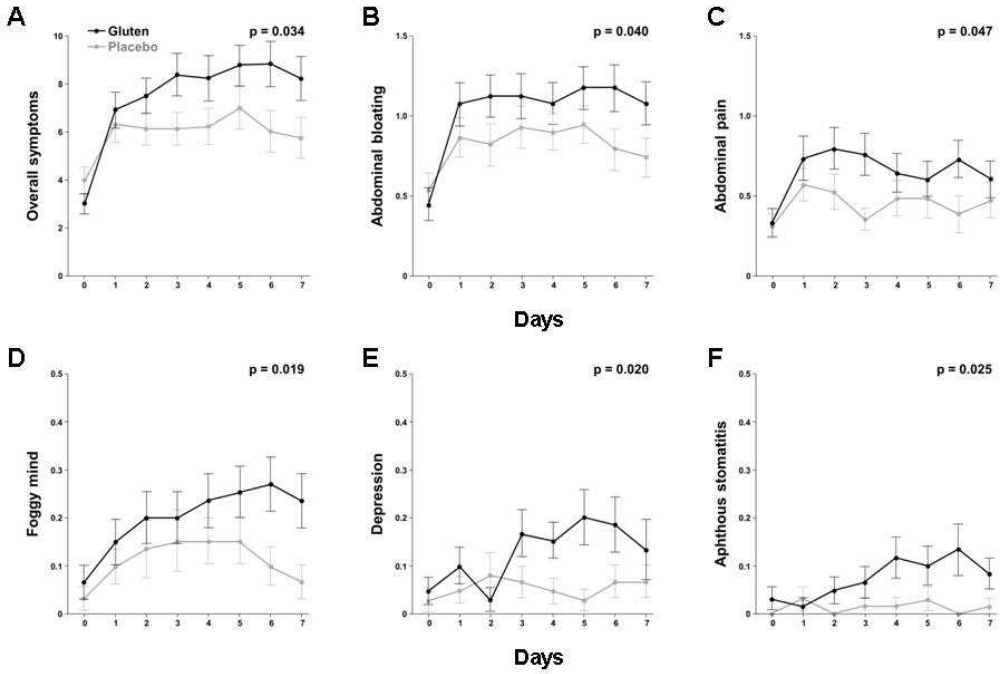


Figure 4

Supplementary Methods

Screening. *Ad hoc* screening included serum determination of IgA anti-transglutaminase and anti-endomysial antibodies, IgG anti-gliadin antibodies (AGA), total IgA and IgE, wheat-specific IgE, upper endoscopy with collection of multiple duodenal biopsies, HLA genotyping, fecal calprotectin and lactose breath test.

Compliance. Patients underwent five outpatient weekly appointments, namely at W_1, W_2, W_3, W_4 and W_5, during which patients provided their filled questionnaires and investigators counted any unused capsules remaining in capsule dispensers (the latter procedure was carried out only at W_2 and W_4). Treatment adherence was high across both treatment groups (median value 98%). Such a level of compliance was maintained throughout the two treatment periods. All the patients showed good adherence to a strict GFD over the 5-week trial, as assessed through a validated dietary questionnaire.[16]

Recognition test. To exclude the possibility of patients being able to discriminate between gluten and placebo capsules, we preliminarily tested capsule recognition in a group of 20 healthy volunteers. On two different days, healthy volunteers were asked to take capsules and then clearly state if the capsules contained gluten or not. We found that healthy volunteers were not able to differentiate the appearance and taste of gluten capsules from those of placebo capsules.

Symptoms. The 15 intestinal symptoms, which patients were asked to grade daily from 0 to 3 (0=absent; 1=mild; 2=relevant, 3=severe and interfering with daily activities) were abdominal pain, abdominal bloating, wind, diarrhea, borborygmus, reduced consistency of stools, increased consistency of stools, constipation, urgency, incomplete evacuation, nausea,

heartburn, belching, acid regurgitation and epigastric pain.[16] The 13 extraintestinal symptoms, which patients were asked to grade daily from 0 (absent) to 1 (present), were tiredness, malaise, headache, depression, anxiety, foggy mind, aphthous stomatitis, paresthesia, arthralgia, myalgia, asthma, rhinitis and skin rash.

Serum and fecal parameters. Serum IgG AGA (upper limit of normal range 50 U/ml) and fecal calprotectin (upper limit of normal range 50 μg/g) were determined by ELISA commercially available kits (both from Eurospital SpA Eurospital SpA, Trieste, Italy). HLA genotyping was performed by using the EU-DQ kit (Eurospital SpA). Intraepithelial lymphocytes were blindly counted by two independent observers (intraepithelial lymphocytes/500 enterocytes) in paraffin wax embedded duodenal sections stained with hematoxylin and eosin. To enhance diagnostic accuracy, sections were immunostained using an anti-human CD3 antibody (A 0452; Dako, Glostrup, Denmark).

Statistical analysis. An analysis of variance (ANOVA) for cross-over design was conducted, with factors *subject* (*sequence* and *subject* within *sequence*), *period* and *treatment* was applied in order to evaluate the difference between gluten and placebo treatments in terms of weekly overall and individual symptom scores. All statistical analyses were performed by two-sided tests and a *p* value less than 0.05 was considered as statistically significant. Statistical analyses were conducted using SAS® Version 9.2 statistical software package.

Supplementary Results

Laboratory parameters, such as serum IgG AGA, fecal calprotectin, intraepithelial lymphocyte density and HLA genotyping, were assessed at baseline that is under gluten-

containing diet. Serum mean (SEM) levels of IgG AGA were 32.3 U/ml (4.9 U/ml). Fifteen among the 59 randomized patients (25%) were IgG AGA-positive. No significant correlation was found between serum IgG AGA levels and *delta* overall score. However, two of the three true gluten-sensitive patients had serum IgG AGA levels over the normal range. The proportion of serum IgG AGA positivity in the remaining 56 patients is 24%. Fecal calprotectin at baseline was normal in all cases. The mean (SEM) percentage of intraepithelial lymphocytes at baseline was 25.1% (6.8%). No significant correlation was found between intraepithelial lymphocytes and *delta* overall score. All the three true gluten-sensitive patients showed a percentage of intraepithelial lymphocytes < 25%. Sixteen of the 59 randomized patients (27%) were HLA-DQ2/DQ8-positive. No significant difference was found in the mean *delta* overall score between HLA-DQ2/DQ8-positive and -negative patients. Two of the three true gluten-sensitive patients were HLA-DQ2-positive.

Supplementary Figure Legend

Supplementary Figure 1. **A.** Distribution of patients according to their weekly gluten and placebo intestinal symptom scores. The closer patients are to the dashed diagonal line, the more comparable their degrees of intestinal response are to either gluten or placebo. The squared area incorporates patients experiencing mild degrees of intestinal response (<90) either with gluten or placebo (46 of 59). **B.** Distribution of patients according to their *delta* weekly intestinal score, calculated by subtracting the intestinal score under placebo from that under gluten. The mean (SD) *delta* weekly intestinal score was 8.6 (38.5) (dashed line). Only three patients had a *delta* intestinal score higher than the cut-off (mean+2SD=85.6).

Supplementary Figure 2. **A.** Distribution of patients according to their weekly gluten and placebo extraintestinal symptom scores. The closer patients are to the dashed diagonal line, the more comparable their degrees of extraintestinal response are to either gluten or placebo. The squared area incorporates patients experiencing mild degrees of extraintestinal response (<40) either with gluten or placebo (50 of 59). **B.** Distribution of patients according to their *delta* weekly extraintestinal score, calculated by subtracting the extraintestinal score under placebo from that under gluten. The mean (SD) *delta* weekly extraintestinal score was 4.7 (15.6) (dashed line). Only three patients had a *delta* extraintestinal score higher than the cut-off (mean+2SD=35.9).

Supplementary Table 1. Sum of scores of the 15 intestinal symptoms experienced during the gluten week by all the 59 patients who completed the study*

Symptom	Score sum
Abdominal bloating	466
Abdominal pain	288
Wind	245
Incomplete evacuation	185
Belching	163
Borborygmus	156
Reduced stool consistency	143
Constipation	135
Urgency	116
Nausea	110
Epigastric pain	99
Increased consistency of stools	88
Diarrhea	67
Heartburn	59
Acid regurgitation	48

*Symptoms are listed in decreasing order from the most to the least scored

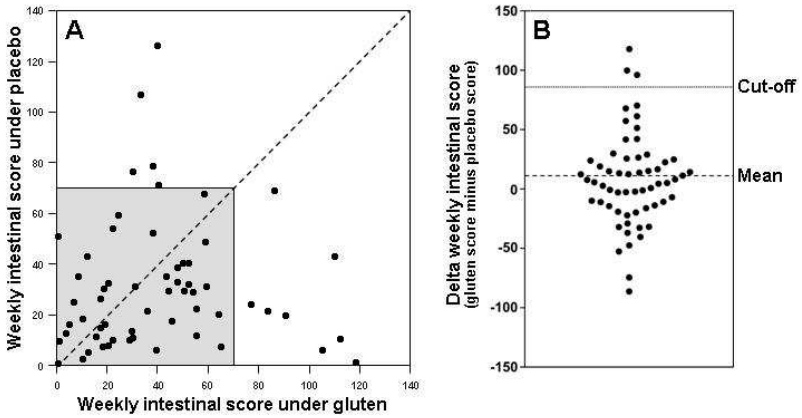
Supplementary Table 2. Sum of scores of the 13 extraintestinal symptoms experienced during the gluten week by all the 59 patients who completed the study*

Symptom	Score sum
Headache	140
Tiredness	131
Malaise	120
Foggy mind	92
Arthralgia	72
Myalgia	63
Skin rash	62
Depression	58
Anxiety	53
Paresthesia	43
Aphthous stomatitis	34
Rhinitis	27
Asthma	12

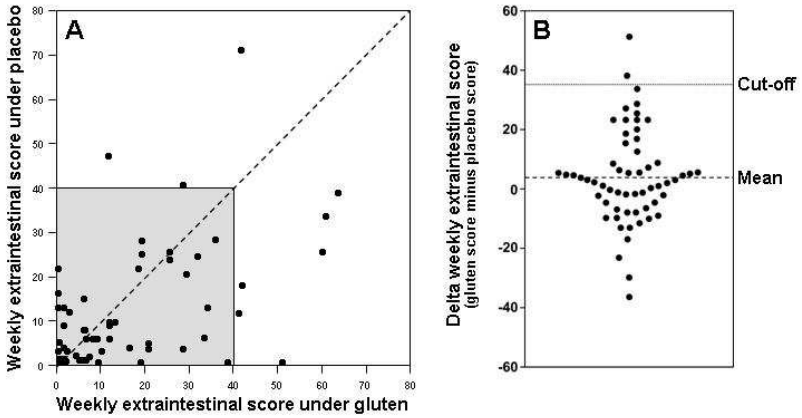
*Symptoms are listed in decreasing order from the most to the least scored

Supplementary Table 3. Prevalence of intestinal and extraintestinal symptoms during the gluten week in the 59 patients who completed the study

Symptom	Percentage
Abdominal bloating	77.9
Abdominal pain	77.9
Wind	69.4
Headache	67.2
Tiredness	55.9
Reduced stool consistency	52.5
Incomplete evacuation	50.8
Malaise	50.8
Constipation	49.1
Borborygmus	47.4
Urgency	45.7
Belching	42.3
Nausea	38.9
Increased consistency of stools	33.9
Foggy mind	33.9
Anxiety	28.8
Heartburn	27.7
Depression	27.1
Epigastric pain	25.4
Diarrhea	25.4
Myalgia	25.4
Acid regurgitation	22.0
Arthralgia	22.0
Skin rash	20.3
Paresthesia	18.6
Aphthous stomatitis	15.2
Rhinitis	11.8
Asthma	3.3



Supplementary Figure 1



Supplementary Figure 2