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Alimentary Tract

"The crackers challenge": A reassuring low-dose gluten challenge in adults on gluten-free diet without proper diagnosis of coeliac disease

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ABSTRACT

Background: Gluten-free diet (GFD) is the one therapy in coeliac disease (CeD). Unfortunately, some patients adopt GFD before the diagnostic work-up. The guidelines suggest a 14-day gluten intake > 3 gr to get CeD diagnosis, although many subjects refuse this approach. Other evidence showed that the intake of 50 mg/day of gluten for 3 months could be useful for CeD diagnosis.
Aims: We performed a dietary study, administering a low dose of gluten in form of "crackers" (about 60–120 mg of gluten/day) for 3 months, to get a final diagnosis of CeD in subjects already on GFD.
Methods: We enrolled adult patients with a suspicion of CeD on self-prescribed GFD. All subjects performed the crackers challenge for 3 months. At the end, all patients were analysed for CeD serology and if positive underwent endoscopy/histology. Also, we recorded the grade of satisfaction for the gluten challenge and the onset of adverse events.
Results: We enrolled 120 patients. All patients concluded the challenge without relevant adverse events. Serological positivity was detected in 54 patients (45%). Histology showed atrophy in 87% and Marsh 1–2 grade in 13% of patients. Ninety-nine patients (83%) were satisfied by this challenge.
Conclusions: The "crackers challenge" is a useful and safe diagnostic approach in people on self-administered GFD.

1. Background

Coeliac disease (CeD) is a chronic, systemic, auto-immune disease precipitated by gluten ingestion in genetically predisposed individuals [1–3].

In gluten-sensitive patients, gluten determines crypt hyperplasia, infiltration of intraepithelial lymphocytes (IELs) and villous shortening of the duodenal mucosa [4,5]. Clinical presentation of CeD is heterogeneous; symptoms include diarrhoea, weight loss, anaemia, abdominal pain, fatigue, but also dermatitis herpetiformis, osteoporosis and infertility [1,2,4,6-8]. The diagnostic work-up includes serology and duodenal biopsies, which are recommended to confirm the disease [5].

Gluten-free diet (GDF) is the only effective therapy for patients affected by CeD, determining positive outcome on histological damage, laboratory tests, clinical symptoms, and complications of the disease [4,5].

Dietary products are considered safe in CD patients when containing less than 20 ppm of gluten (20 mg/kg). However, some patients adopt GFD without proper serology and/or histology. In such a case, the patient needs to be re-exposed to gluten by a useful gluten challenge (GC) that will be conducted with different amounts of gluten and for different times [1,3,5,9], in order to rule out a CD diagnosis.

The British Society of Gastroenterology Guidelines for CeD suggest a 14-day gluten intake ≥ 3 gr (about 2 slices of bread/day), which can induce serological and histological changes in most CeD patients [5]. In 2007, Catassi et al. showed that the intake of 50 mg/day of gluten for 3 months induced villous atrophy and serological positivity in most CeD patients [10]. In a clinical setting, a proper GC with a fixed amount of gluten is rarely possi-

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ble, as there are no 'capsules' containing gluten or cereal bars with fixed amounts of gluten available in the market. Therefore, in most cases, the GC is performed by adding bread or cookies to the GFD for 2–4 weeks. However, many patients refuse taking part in or discontinue these types of GC due to the onset of symptoms or fear of symptom flare. In most cases, the nocebo effect cannot be excluded, and the CeD diagnosis remains vague [11]. In Italy, crackers, also called saltines, are traditionally considered easy-to-digest food, commonly consumed in case of 'stomach-ache' and seasickness, and in special diets. Hypothesising that crackers might overcome the nocebo effect, we observed the outcomes of a long-term GC conducted by administering a low daily dose of gluten in the form of 2–4 regular crackers.

2. Methods

2.1. Study population

This was a prospective study that included all consecutive adult patients (>18 years) with suspected CeD but already on a GFD who were HLA DQ2/8-positive and referred to the Gastrointestinal Unit of either of the University of Naples "Federico II" or the University of Salerno between May 2019 and September 2022. All subjects had adopted self-prescribed GFD before proper CeD diagnostic work-up and refused to return to gluten-containing diets or join the traditional high-dose GC to assess if they had CeD. They were each invited to continue their GFD by adding a half (or full) package of regular crackers daily (2–4 crackers/day) containing 60 mg or 120 mg of gluten for a 3-month period.

All patients with positive serology at time of enrollment followed conventional management for CeD diagnosis (endoscopy and duodenal biopsies) and were not included in the present study. By contrast, all subjects on self-prescribed GFD without a proper serology before commencing GFD, that were seronegative at time of enrollment were included in the study.

Written informed consents were obtained from all participating patients. For each patient we recorded the anthropometry, symptoms/signs of CeD (diarrhoea, weight loss, abdominal pain, anaemia, bloating, osteopenia/osteoporosis, infertility, steatosis, hypertransaminasemia, growth failure, chronic fatigue/asthenia), diagnosis of autoimmune diseases or positive family history, and HLA-DQ2/DQ8 genetic testing.

2.2. Serology

All cases were analysed for serological levels of a-tTG, EMA and DGP 3 months from the start of the crackers challenge.

A-tTG IgA antibodies were measured by ELISA (Enzyme-Linked Immunosorbent Assay, automated system; Delta Biologicals SRL, Rome, Italy), using the human recombinant tTG as an antigen; serum samples with antibody titre higher than 7 U/mL were considered positive. EMA IgA antibodies were assessed by operators confident with the immunofluorescence procedure (Enzyme-Linked Immunosorbent Assay; Delta Biologicals SRL, Rome, Italy). Commercial enzyme-linked immunosorbent assays were used for detection DPG (Enzyme-Linked Immunosorbent Assay; Delta Biologicals SRL, Rome, Italy).

Results were expressed qualitatively as positive or negative findings. All serological evaluations were performed at the laboratories of the University of Naples and the University of Salerno.

2.3. Endoscopy and histology

All patients underwent esophagogastroduodenoscopy (EGDS) with biopsies at 3 or 6 months after the GC, as per the GC guidelines [2,5].

Endoscopists who performed the EGDS were all CeD specialists and were blinded to the results of the serological tests.

Pathologists with experience in CeD diagnosis performed histological evaluation of the duodenal biopsies at the Departments of Pathology of both the University of Naples "Federico II" and the University of Salerno. Histological damage was classified as follows: Marsh I (infiltrative), defined as a condition of normal villous architecture, normal crypts and >30/100 IELs; Marsh II (infiltrative–hyperplastic), defined as a condition of normal villous architecture, crypt hyperplasia and >30/100 IELs; Marsh III, defined as a condition of crypt hyperplasia, >30/100 IELs and mild villous atrophy (Marsh 3A), moderate villous atrophy (Marsh 3B) or total villous atrophy (Marsh 3C); Marsh IV, defined as a condition of atrophy and >30/100 IELs [12].

2.4. CeD diagnosis

Marsh-type histological damage $\geq$ 2 was considered suggestive of CeD, while Marsh 1 was considered suggestive of "potential CeD" if associated with positive serology [5]. Patients who were diagnosed of CeD at the end continued with GFD.

Patients with negative serology continued the challenge for another three months, remaining on GFD and adding a half/full package of crackers daily containing 60 mg or 120 mg of gluten.

After the three months, the serological levels of a-tTG, EMA and DGP of these patients were analysed again. Those who still showed negative results were considered as negative for CeD.

2.5. Adverse events and patient satisfaction

We monitored the possible onset of adverse events (gastrointestinal events, such as bloating, abdominal pain and diarrhoea, and any other adverse events) during the challenge.

We evaluated patients' level of satisfaction with this diet challenge using a simple Visual Analogue Scale (VAS), with a range between 1 and 10. The VAS was a 100-millimetre long horizontal line. The two ends of the line indicated extremes of satisfaction (from absent satisfaction to extreme satisfaction). Each patient graded his satisfaction by making a mark on the line. The exact question was "Are you satisfied with this diet challenge?" [13].

2.6. Statistical analysis

Continuous and categorical variables were reported using medians and interquartile ranges (IQR). Chi-square tests and the Fisher exact tests were used to compare the categorical variables, while the Mann–Whitney U tests and the Kruskal–Wallis tests were used to compare continuous data.

Cohn's k of agreement was used to test the consistency of the different serological tests (a-tTG, EMA, DGP).

3. Results

We proposed the "crackers challenge" to 151 consecutive patients. At the end 31 patients (20%) refused to participate to the trial, so that the study included 120 consecutive HLA DQ2/DQ8-positive patients, with a mean age of 38 years old ($\pm$ 16.1 years), 42 of whom were male and 78 females.

Sixty-three out of the 120 cases (52.5%) reported a family history of CeD. Thirty-two cases out of the 120 (26.7%) were diagnosed with Hashimoto's thyroiditis, and 17 (14.2%) were diagnosed with type 1 diabetes mellitus (DM). Before adopting GFD, 37 patients (30.8%) referred diarrhoea, 30 patients (25%) had weight loss, 49 cases (40.8%) had abdominal pain, 23 subjects (19.2%) had bloating, 2 cases (1.7%) had osteoporosis, 13 patients had infertility (16.7%) and 8 patients had growth failure (6.7%). The laboratory

Table 1
Characteristics of the 120 patients enrolled in the study.

	Number (%)
Demographic	
Male	42 (35)
Female	78 (65)
Mean age ± s.d.	38 ± 16.1
Symptoms	
Diarrhea	37 (30.8)
Weight loss	30 (25)
Abdominal pain	49 (40.8)
Anaemia	51 (42.5)
Bloating	23 (19.2)
Osteoporosis	2 (1.7)
Infertility	13 (16.7)
Steatosis	14 (16.8)
Hypertransaminasemia	26 (21.7)
Growth failure	8 (6.7)
CeD risk factors	
Type 1 diabetes mellitus	17 (14.2)
Hashimoto's thyroiditis	32 (26.6)
Positive family history	32 (26.7)
HLA genotype	
DQ2	101 (84.2)
DQ8	10 (8.3)
DQ2/DQ8	9 (7.5)

S.D.: Standard deviation.

Table 2
Main diagnostic results.

	Number (%)
Laboratory	
a-tTG (U/ml)	51 (42.5)
EMA	49 (40.8)
DPG	48 (40)
Histology	
Marsh 1–2	7 (13)
Marsh 3	47 (87)
Patient satisfaction (VAS > 6)	99 (83)
Adverse Events	35 (29.2)
Bloating	15 (12.5)
Abdominal pain	12 (10)
Mild diarrhea	8 (6.7)
Other	0 (0)

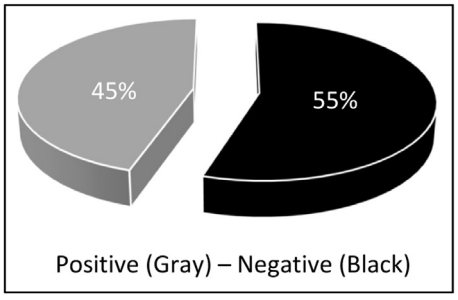

Fig. 1. The percentage of patients with serological activation/positivity after the gluten challenge.

tests showed that 51 patients (42.5%) had anaemia and 26 cases (21.7%) had hypertransaminasemia. Regarding HLA genotype distribution, HLA DQ2 was found in 101 patients (84.2%); HLA DQ8 in 10 subjects (8.3%), while both DQ2/DQ8 were found in 9 patients (7.5%). Main patient characteristics and diagnostic results are reported in Tables 1 and 2.

Three months from the beginning of the crackers challenge, we found serological positivity/activation in 54 (45%) of the 120 patients enrolled in the study (Fig. 1), with evidence of a-tTG positivity in 51 patients (42.5%), EMA (slight/full) positivity in 49 patients (40.8%) and DGP positivity in 48 patients (40%), with a very good agreement both between a-tTG and DGP ($k = 0.86$), and a-tTG and EMA ($k = 0.88$).

The 54 subjects with serological positivity/activation underwent endoscopic evaluation. Histological examination of their duodenal specimens showed the presence of atrophic mucosa in 47 (87%) of these 54 patients and of Marsh 1–2 grade lesions in the remaining 7 (13%). The 66 patients who showed no serological positivity or activation continued the trial for 3 more months, remaining on GFD with a half/one package of crackers every day (60 mg −120 mg of gluten). Three months later, i.e., 6 months from the start of the crackers challenge, these patients again underwent serological evaluation of a-tTG, EMA and DGP. Results of the laboratory tests showed no substantial modifications. Thirty-four of these patients agreed to undergo endoscopy and biopsy; histology showed a Marsh 0 in 28 cases and Marsh I in 6 cases. Finally, these patients were considered as CeD-free.

The other 32 patients refused to undergo endoscopy. Ten of them returned to gluten-containing diets and 22 to gluten-restricted diets with lapses. At one-year follow-up, none of them showed positive serology. However, a conclusive diagnosis could not be reached for these 32 subjects. Fig. 2 shows a summarized flowchart of our study.

No differences in challenge response were found in accordance with HLA status ($p = 0.25$).

During the follow-up, we also evaluated patient satisfaction with this low-dose gluten challenge with a simple VAS (with values between 0 and 10): 99 of the 120 patients (83%), with VAS score > 6, were satisfied and reassured by this dietary approach. Regarding adverse events noted during the study, only 15 of the 120 patients (12.5%) experienced bloating, 12 (10%) experienced mild abdominal pain and 8 (6.7%) experienced mild diarrhoea. All patients could terminate the study without relevant adverse events.

4. Discussion

Data from the present observational study indicate that patients on GFD without proper CeD diagnosis, who refused a short-term high dose of GC, tolerated the low-dose cracker GC well. The cracker GC allowed proper CeD diagnosis in 45% (36% in an intention-to-diagnose analysis including the 31 patients wo refused the trial) of cases and reasonably excluded it in 28%. None of the cases with negative serology that refused endoscopy showed an increase in specific antibodies after 6 months and 1 year of the cracker GC.

Patients complained of no relevant adverse event and even reported their satisfaction with the challenge.

The strengths of our study are that we avoided any possible nocebo effect by utilizing a popular low-cost gluten-containing cracker for the challenge and obtained 100% completion of the GC.

One limitation of our study is that we could not reach any diagnosis in 20% of subjects that refused to participate to the study and in 26% of patients who refused to undergo endoscopy at the end of the cracker challenge. However, the constant negativity of the serology up to one year after the GC supports the hypothesis that the majority does not suffer from CeD. We could not exclude the presence, even if rare, of seronegative CeD patients among them. We are aware that the percentage (45%) of CD diagnosis in our population is quite high. This result probably depends on the high pre-test probability of having CeD of the subjects included in the study. In effect, half of included population had a direct familiarity for CeD, most patients had bowel symptoms, and all were referred to a third-level center for CeD and malabsorptive diseases.

Adopting GFD without completing an accurate work-up for CeD hampers definite diagnosis [9].

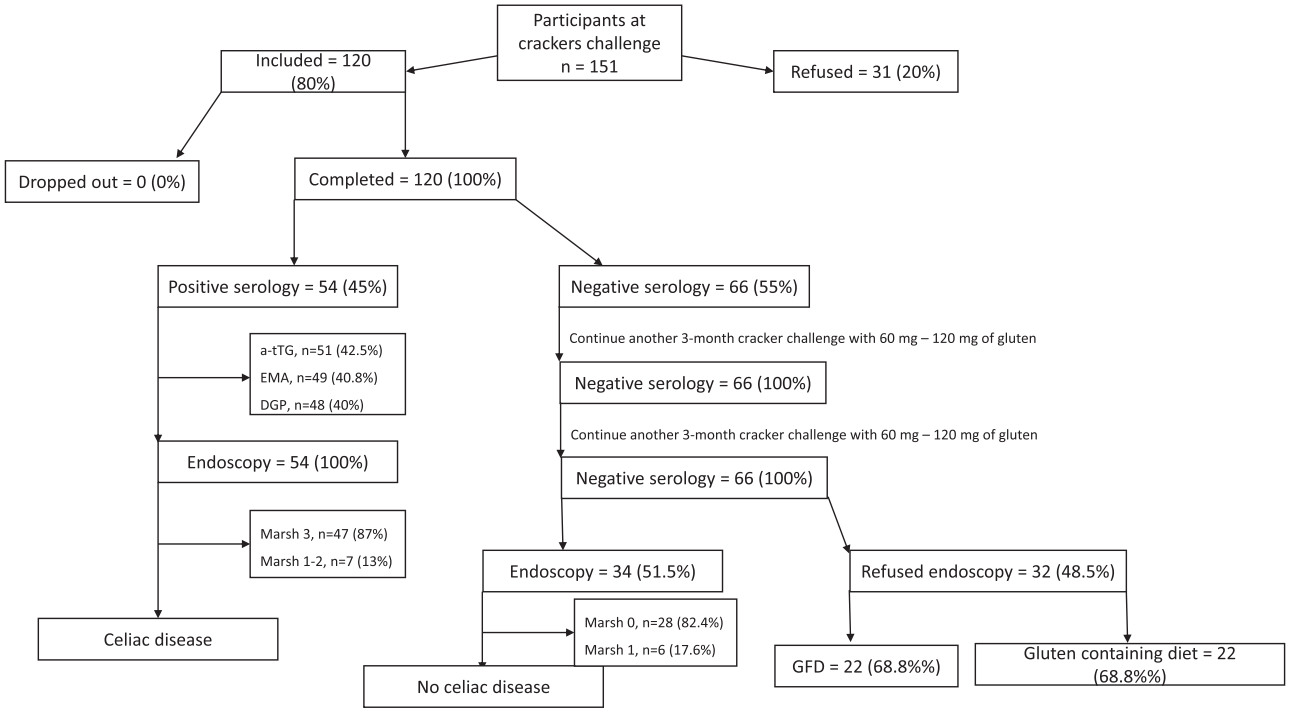

Fig. 2. Flowchart of the study.

However, due to the increasing popularity of GFD, the controversial concept of "gluten sensitivity" and the market push, a non-negligible number of patients adopt GFD before the appropriate serological and histological procedures for CeD diagnosis [6,9]. The reasons are often a family history of CeD and/or symptoms of irritable bowel syndrome (IBS) [6,9]. Most of these patients report improvement in symptoms and wellbeing, but this is a common finding also in IBS and non-coeliac gluten sensitivity. Therefore, evidence of a clinical response after GFD adoption is not a diagnostic criterion specific to CeD [9].

A GFD is defined as an alimentary plan that includes natural or industrialized gluten-free food containing a maximum quantity of 10–20 ppm (20 mg/kg) of gluten [3-6,14].

In a systematic review by Nejad et al., the risk of CeD relapse was estimated to be 0.2% after the consumption of 6 mg of gluten/day, 7% after the consumption of 150 mg of gluten/day, 80% after the consumption of 881 mg of gluten/day and 100% after the ingestion of 150 mg of gluten/day [4].

Several studies confirmed the effect of a minimum amount of gluten on small bowel mucosa, demonstrating that the ingestion of 10 mg/day of gluten was safe in most CeD cases (despite inter-individual variability), but the ingestion of 50 mg/day led to histological damage in half of the patients [10,15].

Another review demonstrated that a daily quantity of 500 mg of gliadin for 4 weeks led to histological alterations in CeD patients [16].

In the past, when serology was nascent and less precise than it currently is, GC was part of CeD diagnosis, as established by the European Society for Paediatric Gastroenterology and Hepatology (ESPGHAN), with demonstration of mucosal lesions, evidence of mucosal healing after adoption of GFD, and relapse after reintroduction of gluten [9,17].

Nowadays, a well-planned GC remains an important cornerstone in cases of diagnostic suspicion of CeD.

However, an internationally validated protocol is still not available in terms of dose, timing or duration of the challenge [9]. Some guidelines, such as the British Society of Gastroenterology Guidelines, suggest a 14-day gluten intake $>$ 3 gr, (two slices of bread/day,) extension of the GC to 8 weeks if serology remains negative at 2 weeks [5].

Leffler et al. confirmed that 3 gr of regular gluten consumption for 2 weeks led to mucosal changes in most CeD patients [18].

Lahdeaho et al. established that a dose of 1–5 gr of gluten caused bowel damage in 67% of patients after 6 weeks, while seroconversion was demonstrated in 43% of patients after 3 months. Catassi showed that the intake of a daily dose of 50 mg/day of gluten for 3 months caused villous atrophy and serological positivity in most CeD patients [19].

In accordance with our findings, a systematic review on the effectiveness and safety of gluten challenge both in paediatric and adult setting found that while symptoms during gluten challenge were hard to predict and had low positive predictive value, serology and mucosal response were more predictable indices of CeD [20].

However, results of gluten challenge regarding mucosal histology in adult patients are variable.

A systematic review by Bruins et al. showed that more than two weeks of a high-dose gluten challenge may be required to induce small intestinal mucosal morphological changes in the majority of patients [20]. However, IEL can appear as early as 1 to 2 days from the start of the gluten challenge, with increased counts in all patients after 4 weeks. Mucosal tTGA-IgA deposit is another marker that appears in the majority of patients within 2 weeks of the challenge [20]. As per serology, in adult patients, the few available studies suggest that no more than half of the patients develop positive serum antibodies (AGA-IgA, EMA-IgA, tTG-IgA, and DGP-IgA/IgG) in response to a 6-week to 3-month gluten challenge.

Interestingly, we found that patients with negative serology who continued the challenge for another three months, remaining on GFD and adding a half/full package of crackers daily containing 60 mg or 120 mg of gluten, did not displayed any clinical and/or biochemical sign of CeD. Therefore, our study demonstrates that a longer challenge with low dose unlikely detects additional CeD patients, thus, may not be worth it to extend. This is different to

challenge with higher amount of gluten, where a late response was shown in only a small proportion of patients.

There is the need for a standardized yet simple GC, in which the quantity of gluten and the length of the GC make it easier for physicians and investigators to evaluate clinical, serological, and histological responses for possible CeD.

In conclusion, the low-dose gluten challenge with crackers or "crackers challenge" is a useful and safe diagnostic approach in people on self-administered GFD. Our pilot study offers a pragmatic, simple, standardized, well-accepted gluten challenge for CeD diagnosis, although prospective and multicentre studies are necessary to further validate this low-dose GC.

Conflict of interest

None.

Author contributions

A.R.: manuscript conception and design, writing & editing of the manuscript, tables and approval; A.D.G., N.I.: writing & editing of the manuscript; M.S., A.S.: data acquisition; S.R., R.D.S., B.T., N.M.C.: actively followed-up the patients; C.C.: critical review of the manuscript, revision, overall supervision and final approval. All authors approved the final version of the manuscript.

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Supplementary materials

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