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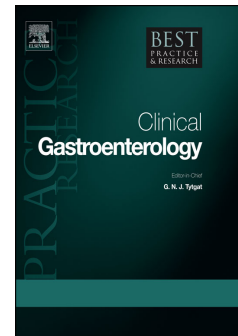
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**Non-celiac Gluten Sensitivity: A Work-in-progress Entity in the Spectrum
of Wheat-related Disorders**

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Abstract

Non-celiac gluten sensitivity is an undefined syndrome with gastrointestinal and extra-intestinal manifestations triggered by gluten in patients without celiac disease and wheat allergy. The pathogenesis involves immune-mediated mechanisms requiring further research. Symptoms disappear in a few hours or days after gluten withdrawal and recur rapidly after gluten ingestion. Besides gluten, other wheat proteins as well as fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs) may contribute to this syndrome. This syndrome occurs mainly in young women, being rare in children. Its prevalence ranges from 0.6% to 6%, based on primary or tertiary care center estimates. No biomarker is available, but half of patients tests positive for IgG anti-gliadin antibodies, which disappear quickly after gluten-free diet together with symptoms. Also, genetic markers are still undefined. Although currently limited to a research setting, double-blind, placebo-controlled, cross-over trial strategy is recommended to confirm the diagnosis. Treatment is based on dietary restriction with special care to nutrient intake.

Key words: Celiac disease; Duodenal biopsy; Anti-gliadin antibodies; Innate / adaptive immunity; Irritable bowel syndrome; Wheat allergy

Introduction

Until a few years ago gluten and wheat proteins were considered responsible for the occurrence of two well-defined clinical entities, i.e. celiac disease (CD) and wheat allergy (WA) [1, 2]. The evidence of gluten sensitive patients, i.e. symptoms evoked by gluten/wheat in patients without CD or WA, was brought up for the first time at the beginning of the '80s by Geoffrey Holmes' group in the UK [3]. That remarkable report highlighting the existence of gluten sensitivity fell into shadow as most clinicians were dissatisfied by the lack of established diagnostic criteria to identify those patients. Likewise functional bowel disorders, most subjects with a suspicion of gluten sensitivity were thought to be imaginary (i.e. 'psychiatric') patients belonging to a sort of 'no man's land' of gluten related disorders [4]. Nonetheless, with the increasing number of patients complaining of symptoms following gluten ingestion, the awareness of a new gluten-related syndrome (currently referred to as non-celiac gluten sensitivity, NCGS) grows with a progressive pace over the years [5-9].

Why NCGS has markedly increased over time? The answer(s) to this question is / are still unclear, although a number of factors should be considered. First, the novel variants of wheat that are known to contain high amount of toxic gluten peptides potentially harmful to the integrity of the enteric mucosa; secondly the mechanization of farming and the growing industrial use of pesticides; thirdly, the overall reduced time of dough fermentation resulting in a higher content of toxic gluten fractions in bakery products [10]. Finally, the media hype and the growing awareness of clinicians towards this condition contributed to the increasing rate of NCGS diagnosis, not always supported by evidence [11].

Currently, NCGS should be regarded as a new entity although with a possible overlap with other conditions, i.e. mainly functional bowel disorders (irritable bowel syndrome, IBS) and a variety of food hypersensitivities [12]. In this respect, dietary components other than gluten, such as wheat proteins (i.e. amylase- and trypsin-inhibitors - ATIs) and fermentable oligo-, di-, mono-

saccharides and polyols (FODMAPs) could have a role in symptom generation of NCGS [13, 14]. However, the relative contribution exerted by single dietary factors in NCGS-related symptoms is still unclear as cereals contain both ATI and FODMAPs admixed with gluten. NCGS should be suspected in patients displaying intestinal symptoms (often dismissed as 'IBS patients') and extraintestinal manifestations, which occur soon after gluten ingestion [15, 16]. A diagnostic prerequisite for establishing NCGS in a symptomatic patient is that both CD and WA have been ruled out. Due to the lack of biomarkers, the diagnosis of NCGS remains strictly related to clinical criteria [17, 18]. The improvement of symptoms after gluten exclusion is regarded as one of the major diagnostic criteria for NCGS, although the possibility of a 'placebo effect' (associated to a dietary exclusion of certain foods) should not be discarded [19]. Thus, a cautionary approach in recommending a gluten free diet (GFD) should be adopted in symptomatic patients lacking a clear diagnosis of NCGS [20].

This chapter has been designed to provide a practical guidance in the various aspects related to NCGS, i.e. basic immunological mechanisms and pathogenesis, epidemiology and clinical picture, diagnostic criteria, treatment (including a possible approach by enzyme therapy) and future tasks, in order to provide specialists and general practitioners with a most updated review on this emerging topic.

Basic immunological mechanisms and pathogenesis

Gluten is an essential triggering factor in CD. However, the term “gluten” is complicated, because there are at least three different meanings of the word. A gluten-free diet is a diet without wheat, rye and barley (and all related cross-bred cereals). Gluten is also the glue-like mass obtained after washing flour with water, and denotes a mix of gliadin and glutenin proteins from wheat and related proteins derived from other gluten-containing cereals. In plants, these proteins serve as storage for amino acids and energy for the seeding sprouts. However, the cereals also carry a range of other proteins with similar properties to gluten, which co-precipitate with gliadins and glutenins

during purification. This will be the focus of another chapter in this issue (by prof. Schuppan and co-workers) and some of this work has been previously published [13]. Anyway, it is important to note that a wide range of proteins can, in fact, lead to the effects of “gluten” in the diet. Furthermore, as will also be discussed later, it is possible that non-protein moieties (e.g. FODMAPs) in bread are responsible for the reaction in NCGS – adding further complexity to the overall picture [21, 22].

Gluten has been shown to cause a range of effects in cell culture systems. Some of these effects are due to complex protein mixtures, others to purified proteins, and at least a subset of these effects have been duplicated with synthetic peptides. Gluten has long been known to have effects on several cell lines (K562 cells, Caco-2 cells, macrophages) and focus has been paid to a certain gliadin peptide (spanning 31-43 of alpha-gliadin), although the cellular receptors for these effects are rather unclear [23]. The chemokine receptor CXCR3 is involved in the effects on intestinal permeability and zonulin release [24]. The transferrin receptor CD71 is another interesting candidate for the effects of gliadin peptides on epithelial cell permeability [25]. Recent work suggests that a pepsin digest of gluten can activate peripheral blood mononuclear cells and monocytes via toll-like receptor-2 (TLR2) and TLR4 signalling and thereby activate the MyD88/TRIF/MAPK/NF- κ B pathway [26].

The overall picture of CD immunology is complex, but has reached a level of understanding far beyond that within the field of NCGS. As will be discussed in another chapter in this issue (see chapter 4, du Pré and Sollid), it is clear that adaptive immunity has a crucial role in the pathogenesis of CD [27]. This immunological reaction focuses on intestinal T-cell recognition of gliadin peptides presented by the HLA-DQ molecules that are associated with CD. To the best of our knowledge, such intestinal T cells have not been isolated from any NCGS patient – although it should be realized that systematic attempts might not have been undertaken. The lack of gluten-reactive T cells, and the lack of association between HLA and NCGS together suggest that the adaptive

immune system in NCGS does not act in similar manner as seen in CD. Also, innate immunity has been claimed to have a role in the development of CD, particularly at the beginning of the pathogenic process. Innate immunity can contribute to the pathogenesis of NCGS – but also this has not really been well studied.

Of particular interest are the findings that gluten in the diet can have profound effects on the animal models of autoimmunity, for instance non-obese diabetic (NOD) mice [28, 29]. These mice develop a small intestinal enteropathy with elevated mucosal levels of pro-inflammatory cytokines when exposed to dietary gluten, but the enteropathy is not considered equivalent to that seen in human CD [30]. For instance, serum levels and mucosal expression of anti-transglutaminase antibodies are not gluten-dependent [31]. In NOD mice, dietary gluten alters the composition of their gut microbiome [32], intestinal regulatory T cells [33], as well as their natural killer cell cytotoxicity [34, 35]. The significance of these findings in NOD mice to the human situation needs further investigation.

Although the pathogenesis of NCGS is still unclear, a role for innate, rather than adaptive, immunity has been postulated in NCGS based on the increased small intestinal expression of TLRs, particularly TLR2 and, to a lesser extent, TLR1 and TLR4 [36]. However, signaling through these TLRs has been not yet elucidated. In the same time, adaptive immunity markers, i.e. interleukin (IL) 17A, IL-6, interferon (IFN)- γ , IL-17 and IL-21 were not over-expressed in the duodenal mucosa of NCGS patients. More recently, however, increased levels of IFN- γ have been detected in small intestinal biopsies of NCGS patients after a short gluten challenge, thus supporting a possible pathogenic role for adaptive immunity in this syndrome [37]. According to the latter findings, the detection of anti-gliadin antibodies (AGA) in more than 50% of NCGS patients provides further evidence that adaptive immunity might contribute to NCGS development [38].

Discordant data have been reported on the epithelial barrier function in NCGS. Preliminary studies demonstrated a reduced intestinal permeability in NCGS, thus reinforcing the concept of an

increased intestinal barrier function in this syndrome. In support of this hypothesis a significantly higher expression of claudin-4 (CLDN4) mRNA, a marker of reduced permeability, was found in duodenal biopsies of NCGS patients [36]. Unlike earlier data, subsequent studies found an increased intestinal permeability in a subgroup of NCGS patients with IBS-D carrying the HLA-DQ2+/DQ8+ [39]. This discrepancy implies that further research is necessary to define small intestinal permeability in NCGS.

In addition, changes of the gut microbiota might favour the inflammatory response to gluten [40]. Finally, a further important aspect linking NCGS to IBS is that HLA-DQ8 transgenic mice sensitized by gliadin display an increased secretion of acetylcholine from the myenteric plexus and this results in an enhanced muscle contractility and epithelial hypersecretion. Gluten withdrawal reversed both abnormalities [41].

Epidemiological and clinical picture

Epidemiological data on NCGS are still scant and approximate. In the US this syndrome seems to be observed much more frequently in tertiary referral centers compared to primary care. Data from the National Health and Nutrition Examination Survey (NHANES), a primary care program, based on interviews, physical examination and blood samples, showed that NCGS prevalence was 0.6% over 7,762 subjects in the 2009-2010 period [42]. The low prevalence of NCGS detected in the NHANES study might be ascribed to the questionnaire that was not originally designed for identifying NCGS patients. Conversely, at the Celiac Disease Center (University of Maryland) 6% over 5,896 subjects were identified as NCGS in the 2004-2009 period [5]. Although it is likely that a higher number of NCGS patients are referred to specialized centers, this cannot explain the huge difference observed in these two studies. Based on these data, it has been hypothesized that NCGS might be up to six times more frequent than CD, whose prevalence is generally acknowledged to be 1% worldwide [43].

An Italian multicenter prospective survey compared the prevalence of NCGS and CD diagnoses in 38 referral pediatric and adult centers of gluten-related disorders [44]. NCGS and CD were diagnosed in 391 (3.2%) and 340 (2.8%), respectively, in 12,255 patients consecutively studied in one-year period (from November 2012 to October 2013). Compared to the previously mentioned data [5], these results lower of about 50% the prevalence of NCGS in tertiary care centers. Therefore, it can be assumed that the ratio between NCGS and CD is approximately 1 to 1, a figure that is similar to that reported in the NHANES survey [42]. Although diagnosable at any age, NCGS appears to occur more frequently in adults with a mean age at onset around 40 years. Likewise to functional bowel disorders, the female gender is more prone to gluten sensitivity with a female / male ratio of 5:1 [44]. So far it is rare in pediatric age with a prevalence of less than 3% in 391 NCGS patients [44]. Although NCGS is rare in children, data indicate that gluten avoidance is very common among toddlers in New Zealand (up to 5%), mainly due to non-specific abdominal complaints [45].

NCGS can be regarded as a syndrome rather than a unique disorder, being characterized by a wide array of symptoms occurring soon (i.e., from hours to few days) after gluten ingestion. Usually, symptoms evoked by gluten disappear or improve after gluten withdrawal and recur upon gluten re-challenge [5, 38]. The clinical presentation is a combination of gastrointestinal symptoms and extra-intestinal manifestations [44]. Most of the gastrointestinal symptoms are virtually overlapping to those reported by IBS patients and, according to Rome III criteria, includes abdominal pain / discomfort, bloating (87%) and bowel habit changes: diarrhea (detected in up to 54%), alternating bowel (27%) and constipation (24%). Notably, IBS clinical profile is characterized by frequent fluctuations of symptom exacerbation along with quiescent phases, while gastrointestinal manifestations in NCGS appear to be rather stable over time. Among patients with the overlapping NCGS and Rome III-based IBS (about 50%), one-third showed breath test proven lactose or, more rarely, fructose intolerance [7, 44].

Upper gastrointestinal manifestations identifiable in NCGS patients include epigastric pain (52%), nausea (44%), aerophagia (36%), gastroesophageal reflux disease (32%), and aphthous stomatitis (31%) [44]. The most frequent extra-intestinal features of NCGS include fatigue and lack of well-being together with neurological symptoms, i.e. headache, “foggy mind”, arm / leg numbness and anxiety / depression. Other systemic signs are joint / muscle pain resembling fibromyalgia, skin rash / dermatitis, weight loss, iron-deficiency and / or folic acid anemia and, although rarely, allergic manifestations such as asthma and rhinitis. In young patients GI symptoms, including abdominal pain / discomfort, bloating and diarrhea are more frequent than extra-intestinal manifestations, characterized by fatigue, headache and limb pain [46].

More than 20% of NCGS patients show allergy to one or more inhalants, foods or metals, the most common being mites, *graminaceae*, *parietaria*, cat / dog hair, shellfish and nickel [44]. Among psychiatric disorders, a minority (6%) of patients with NCGS showed a previous clinical history of eating behavior abnormalities, while autism spectrum disorders (ASD) have been hypothesized to be associated with NCGS [47, 48]. Notably, a gluten- and casein-free diet might have a positive effect in improving hyperactivity and mental confusion in some patients with ASD. This very exciting association between NCGS and ASD deserves further study before conclusions can be firmly drawn. Conversely to CD, it remains unsettled whether NCGS patients are at risk for associated autoimmune disorders. Some studies suggest that the finding of autoimmune disorders in NCGS would be a rare association [5, 7, 38].

Based on pathogenetic mechanisms and clinical features, complications known to occur in CD (i.e. ulcerative jejuno-ileitis, collagenous sprue, small intestinal lymphoma and other gastrointestinal neoplasms) are unlikely to be observed in NCGS [5, 49]. Nonetheless, NCGS is still an emerging condition and its natural history as well as occurrence of complications deserves further data before being thoroughly understood.

Diagnostic criteria

The identification of NCGS starts when patients seek medical advice as a result of symptoms / manifestations which are clearly associated with gluten ingestion still present in the diet. According to the diagnostic criteria established by two Consensus Conferences (London 2011 and Munich 2012), the current view to NCGS diagnosis is based on symptom / manifestation evaluation along with the exclusion of CD and WA [5, 7]. The latter should be established by: normal levels of IgA tTGA, IgA EmA and IgG deamidated gliadin antibodies in the absence of villous atrophy at small intestinal biopsy for CD [1]; normal levels of serum IgE antibodies to gluten proteins and wheat fractions as well as lack of skin reaction to Prick tests for WA [2] both sets of exams being performed in patients under gluten containing diet. In the clinical practice, it is very common to see patients on a GFD because of a self-diagnosed NCGS. In such cases a gluten challenge should be suggested for a period of at least 2 weeks in order to rule out CD [50, 51]. Since patients on GFD are reluctant to resume a gluten containing diet because of symptom recurrence, the assessment of HLA-DQ2 and -DQ8 can be a valuable test to rule out CD (Figure 1) [16, 52]. In contrast to CD, NCGS did not show any correlation with HLA-DQ2 and / or -DQ8, although two studies have reported that the prevalence of DQ2 and / or DQ8 in NCGS was slightly higher than that in the general population [5, 38]

A recently proposed approach to NCGS diagnosis is an objective improvement of gastrointestinal symptoms and extra-intestinal manifestations assessed through a rating scale before and after GFD. Although a standardized symptom rating scale is not yet applied worldwide, a recent study indicated that a decrease of the global symptom score higher than 50% after GFD can be regarded as confirmatory of NCGS (Table 1) [53]. However, since the improvement of the clinical picture after GFD can be induced by a placebo effect, a double-blind, placebo controlled challenge (DBPC) is highly recommended to confirm NCGS [16]. Although objective, this approach is still expensive and time-consuming and therefore its use has been confined to a research, rather than

clinical, setting. So far, the few DBPC trials performed to confirm NCGS have produced discrepant results mainly for a possible bias related to patient selection (often not fulfilling the criteria of NCGS) and blindness bias (patients recognizing gluten vs. placebo by the taste of food) [3, 22, 54, 55]. Two of these studies were performed by the same group: one initially confirmed the existence of NCGS through the significant recurrence of symptoms in patients under gluten containing diet [54]; the other showed that gluten was not responsible for symptoms re-occurrence, whereas a low-FODMAPs diet induced a significant symptom improvement in a significant portion of the patients [22]. A third DBPC study revealed that gluten determined the occurrence of intestinal symptoms in IBS patients [55]. The role of gluten as a specific trigger of this new gluten-related syndrome has been recently confirmed by a DBPC cross-over trial, performed in 61 patients with a large panel of gastrointestinal (including IBS-like) and extra-intestinal symptoms / manifestations remitting after GFD [56]. The novelty of this study was the use of gluten-containing capsules, which were indistinguishable from placebo capsules in terms of taste and appearance. A daily dose of 4.375 g of gluten (or placebo) was administered for one week followed by a one-week wash out before crossing over to one week of the other type of capsules. It is relevant to underline that the amount of gluten used in this trial was significantly lower than that administered in the previous DBPC (13 - 20 g). Gluten ingestion evoked a significantly higher severity of overall symptoms than placebo ($P=0.034$), particularly evident for bloating ($P=0.040$), abdominal pain ($P=0.047$), aphthous stomatitis ($P=0.025$), foggy mind ($P=0.019$) and depression ($P=0.020$). However, the analysis of the individual patients' overall scores showed that only some patients had an evident worsening of symptoms after gluten ingestion. This could imply that their clinical gluten sensitivity could be caused by other triggers, such as ATIs and FODMAPs, as well as non-dietary (e.g. psychological) factors, leading to similar responses to gluten and placebo.

AGA can be sought in NCGS patients as possible diagnostic adjunct. In this line, recent data showed that about half of NCGS patients tested positive for the 'classic' AGA [5, 38, 55]. In NCGS patients these antibodies are mainly IgG class (only occasionally IgA) and are consistently

associated with very low titers of IgA tTGA (< 1 arbitrary units) in absence of IgA deficiency [38]. In contrast to CD, where AGA IgG persisted in at least 50% of patients, the result of one study demonstrated that almost in all patients with NCGS AGA became undetectable within 6 months of GFD [53]. These findings are in line with the possibility that an immunological memory might be active in CD but not in NCGS [57]. Other laboratory tests can be helpful to identify signs of malabsorption, which occurs in 20-30% of patients with NCGS. Abnormalities include low levels of ferritin, folic acid, vitamin D-25-OH and vitamin B₁₂ [44]. In some cases these absorption abnormalities improve after GFD. A slight increase of stool calprotectin, an indicator of intestinal mucosa inflammation, can be found in NCGS. Cytometric basophil activation has been demonstrated *in vitro*, an interesting finding that implies food allergy at least in a subset of NCGS patients [55]. In line with vitamin D-25-OH malabsorption it has been recently reported that about half of NCGS patients shows bone mass depletion [58]. This was found to be closely related to a low dietary intake of calcium and to a low BMI, mainly in NCGS patients with other food (i.e. lactose) intolerance. Dietary support should be strongly recommended at the time of NCGS to prevent the occurrence of osteopenia.

Duodenal biopsy is also a very important step in the diagnostic approach to NCGS particularly to rule out definitively an underlying seronegative CD in symptomatic patients (Consensus Conferences 2011 e 2013). Likewise serology, the duodenal biopsy should be obtained on a gluten containing diet. Although small bowel villous architecture is normal in NCGS, the number of intraepithelial lymphocytes (IELs), defined normal if $\leq 25\%$ [59], is increased in a high proportion of patients [36-38, 44]. Notably, the increase of IELs found in NCGS is significantly less pronounced than that detected in CD, thus suggesting the existence of a mucosal low grade inflammation [36].

This mild lymphocytic intestinal infiltration can result in a specific NCGS histological pattern, characterized by a linear distribution of CD3⁺ T lymphocytes in the deeper part of the

mucosa together with clusters of the same cells in the superficial epithelium [60]. Differently from CD, in NCGS there is no increase of T-cell receptor γ/δ IELs [36]. Although small intestinal mucosa in NCGS shows a normal villous / crypt ratio ($>3:1$), it is generally acknowledged that a mild inflammation may contribute to impair intestinal absorption [16].

Mucosal and peripheral cytokine responses have been investigated in NCGS patients. Increased IFN- γ mRNA, albeit at a low level, after short term (three days) gluten challenge was observed in NCGS patients [37]. Higher levels of interleukin-10, granulocyte colony-stimulating factor, and transforming growth factor- α were produced by peripheral blood mononuclear cells (PBMC) of IBS patients in response to gluten compared to rice [39]. Recently, an overproduction of the pro-inflammatory chemokine CXCL10 from PBMC obtained by NCGS patients in response to various cereal fractions was demonstrated [61]. Increased CXCL10 secretion was significantly related to the stimulation of PBMC from NCGS patients by gluten-containing cereal proteins and not by corn and rice flours. Moreover, by analyzing the effect of modern wheat varieties (*Manitoba* and *Claudio*) with that of ancient varieties (*Senatore Cappelli* and *Khorsan*), it has been demonstrated that modern wheat induced a significantly higher secretion of CXCL10 by PBMC. The analysis of the in vitro response of PBMC from CD patients showed a ten-time higher secretion of CXCL10 compared to NCGS patients, whereas treated CD patients did not produce this chemokine with a similar behavior to healthy controls. These results suggest that CXCL10 might be a diagnostic marker of NCGS.

Dietary treatment

Patients should avoid self-diagnosis and should not withdraw gluten by themselves before CD or NCGS condition has been verified in a referral center [16]. The auto-diagnosis of a presumed gluten intolerance is a problem since once GFD is started many find it is difficult to reintroduce gluten to make it sure that the patient has not CD [62]. However, as long as CD is adequately

eliminated, the patient can start GFD. Most patients referred to the dietitian with suspicion of NCGS are already self-instituted on a GFD. The dietitian will complete a nutritional assessment, evaluate diet adherence and educate in GFD. The dietitian educates in how to keep food diversity and nutritional value in a restricted diet. Issues that are addressed are balanced meal planning, fibers and enrichments, hidden gluten sources, label reading and additional food allergies or intolerances. After the confirmation of NCGS diagnosis, according to the previously mentioned work-up, patients are advised to start with a GFD [49].

What is gluten-free diet ?

GFD is based on the elimination of the alcohol-soluble protein fractions of gliadin in wheat, secalin in rye and hordein in barley, that are all toxic in CD. The cereals are replaced by gluten-free cereals such as rice, corn, buckwheat and millet. Moreover, some leguminosae such as quinoa, amaranth and soybean are particularly useful due to their high protein content quality as replacements for their gluten containing analogues. However, as recommended for celiac patients, a diet mainly based on naturally gluten free cereals and non-processed food (meat, fish, poultry, egg, fruit, vegetables) facilitate GFD adherence and secure the nutritional value of the diet. Oats that are not contaminated with wheat (requires dedicated supply chain and manufacturing) have shown to be safe in a GFD in CD [63, 64] and are allowed as part of GFD in most countries. In CD, intestinal mucosal healing is dependent on a strict GFD. In NCGS, however, the health benefit from GFD seems to be mostly symptomatic, and so the degree of diet strictness is defined by individual tolerance, as goes also for the use of oat.

So far, it is not yet established whether NCGS patients must avoid potential cross-contamination of gluten. However, it is reported that some patients with suspicion of NCGS can have symptoms even after consumption of small amounts of gluten [5]. In CD the safe level of gluten intake is often referred to as “less than 50 mg / day”, although a safe limit might be from 10 to 100 mg / day [51]. There is no defined limit for gluten intake in NCGS.

The nature of the GFD has changed over time. The new dual codex standard for gluten-free, adopted by EU in 2009, reduced the amount of gluten in “gluten-free” labeled foods from 200 to 20 mg parts per million according to the Food Standards Agency emanated in 2005. GFD became popular in the last few years, and the global gluten-free market has increased substantially [65]. The liberalization of uncontaminated oat [66] and the use of pseudocereals as replacement for refined gluten-free flours have increased both the food variety and the nutrient content of the GFD [67, 68].

Still, reports and dietary clinical practice show that GFD differs substantially from gluten containing diet because of a higher content of fat and lower content of protein and fiber [69]. Two recent studies indicated nutritional inadequacies of folate, calcium, iron and zinc in both recently diagnosed and long-term patients with CD [70, 71]. Inadequate intake did not relate to nutrient density of the GFD, indicating the importance of dietary education [69]. There is a need to define the recommended level of gluten exclusion, as well as its long-term consequences in NCGS patients.

Dietary adherence

Some attempts have been made to estimate adherence to GFD in CD patients. A review of Hall and coworkers showed strict adherence rates, from 44% to 90%, measured by professional assessment [72]. The major problem in estimating GFD adherence resides in its many definitions and the lack of standardized methods how to measure the actual patients' compliance to diet. The gold standard method for assessing adherence to GFD in celiac patients is still an expert dietitian evaluation [73]. Two different tools based on self-administered questionnaire have been developed [73, 74], but the value of these tools remains to be investigated further.

To our knowledge, there is no information on GFD adherence in patients with NCGS. Research in progress from our group show no significant difference in GFD adherence between CD and NCGS (unpublished data). AGA of the IgG class have been suggested as a possible marker of compliance in NCGS patients, since these antibodies disappear in a few months together with symptom resolution [53].

Nutrient status

Compared to CD patients, nutritional status at diagnosis and follow-up is not well described in NCGS patients. In contrast to CD patients, who may be malnourished, NCGS patients do not usually display signs of malnutrition at the time of diagnoses. Deficiency of fat-soluble vitamins (e.g. vitamin D), B12, folate, iron and calcium are not uncommon in CD patients, and these parameters should be tested in all CD patients in a yearly follow-up according to guidelines from the British Society of Gastroenterology [51]. In a study of 276 NCGS patients diagnosed with DBPC challenge weight loss was found in 35% of cases as compared to CD (52%). The same difference was seen for anemia, the frequency being 24% and 78% in NCGS and CD patients, respectively. Although less prevalent than in CD, weight loss and anemia in NCGS patients raise the question whether these findings are a consequence of malabsorption or dietary restriction [55].

Another report from the same authors revealed that 47% of NCGS patients showed osteopenia or osteoporosis, as compared to 64% in CD patients [58]. The frequency of other micronutrient deficiencies in NCGS is not well described and there are no guidelines for follow-up on nutritional status. In the Volta et al. study laboratory tests showed low ferritin and vitamin D levels in 23% and 11% of NCGS patients, respectively [44]. In conclusion, a cautionary approach should be adopted before concluding on nutritional deficiencies in NCGS patients at the time of diagnosis.

Another important issue to consider is to maintain a good nutritional intake in the diet over time. All long-term exclusions of important food items should be professionally monitored to reduce risk of malnutrition. Evaluation by a dietitian should be a part of the follow-up for NCGS patients [75].

Management of patients with persistent GI-symptoms

About one third of patients with self-reported NCGS complain of persistent symptoms while on a GFD, a figure comparable to that found in treated CD [17, 76]. This observation might have several explanations. In addition to wrong diagnosis of NCGS, a poor compliance to diet is the first option that should be considered. In the Biesiekierski et al. study only 58% of self-diagnosed NCGS patients reported adherence to GFD [17]. As these patients do not suffer from CD, they may feel they do not need to follow a strict GFD, although NCGS patients can experience more symptoms than CD patients following a short gluten challenge [77]. NCGS patients may also unintentionally eat gluten and this can only be revealed through expert dietitian evaluation. In addition to gluten, another cause of persistent symptoms is multiple food intolerance / hypersensitivities. The next step is therefore to identify possible dietary factors triggering symptoms, e.g. FODMAPs or oat. Increasing evidence shows that FODMAPs e.g. milk, ice cream, apple juice, onions, garlic, honey, legumes and sorbitol/xylitol-sweetened food can cause symptoms in patients with IBS [22]. A systematic elimination and re-introduction of suspected items, guided by dietitians, is necessary to confirm the diagnosis of FODMAP intolerance [78]. Oats are of high nutritional value and are generally recommended and regarded as safe in a GFD. Previous data showed that some CD patients developed enteropathy when oat was included in the diet [79, 80]. The risk of enteropathy in CD patients may depend on oats varieties [66]. In contrast, the role and frequency of oats intolerance remain to be investigated in NCGS patients.

Enzyme-treatment

Gluten-specific proteases, called glutenases, produced by bacteria and fungi, might digest gluten in small fragments, thereby reducing its toxicity for the small intestinal mucosa [81]. A phase 2 clinical trial showed that these enzymes can attenuate “in vivo” the gluten-induced small intestinal injury in patients with CD in a GFD containing up to 2 g gluten daily. This approach is not going to replace GFD for the treatment of CD, but it could be very useful to protect CD patients from the risk of contaminations. Once validated, this enzyme therapy could be useful for the treatment of low-threshold NCGS patients.

Life-long diet ?

It is still unclear whether NCGS is a permanent or transient condition [5, 7, 82]. Only a few patients with NCGS reintroduce gluten in their diet after the significant improvement achieved by GFD, because they fear the symptoms will return. Theoretically, an attempt to reintroduce gluten after 1-2 years on GFD might be advised. A proposed approach might be a desensitization trial with a progressive introduction of small amounts of gluten [83].

Future tasks

A wide spectrum of clinical and research tasks represent future objectives in the field of gluten-related disorders, in particular for NCGS. The increasing number of patients who experience symptoms after gluten ingestion without evidence of CD and WA has contributed to the recognition of NCGS as a new gluten-related disorder [5, 7, 16]. Although the existence of NCGS has been accepted worldwide, the knowledge regarding this syndrome is still limited, with many aspects awaiting further definition [6].

The first challenge is to provide clinicians with solid criteria through which establish a diagnosis of NCGS. In the future, we think a two-step approach will be recommendable, namely 1) identification of suspected patients by showing a significant improvement of gastrointestinal and extra-intestinal symptoms as a result of the GFD. In line with this, symptom score scales will represent a mandatory research objective to assess symptoms and their response to GFD; 2) prove the diagnosis using DBPC trial with gluten. While waiting for a valuable biomarker of NCGS, the only diagnostic strategy to confirm NCGS remains the DBPC trial. However, many aspects of DBPC deserve standardization before being applied in the clinical practice. The first issue is related to the unavoidable taste of gluten, which may influence patients and generate symptoms (i.e., 'nocebo-effect'). This issue can possibly be overcome by the use of the muesli bars, which avoid recognition of the presence of gluten. In order to overcome the awareness of gluten by the patient,

recently gluten has been administered in capsules. However, since capsules may contain 0.5-0.8 gr of gluten, their number / day may be quite high (>10), thus hindering the patients' compliance. Secondly, the lack of a standardized dose of gluten to be administered in DBPC trials limited the interpretation of the results and therefore the diagnosis of NCGS. Specifically, how much gluten is necessary to evoke symptoms? This answer is still undefined as reflected by the wide array of doses of gluten so far administered ranging from 4.375 to 20 g / day [3, 22, 54-56]. Thirdly, because of the heterogeneity of the experimental protocol, it is essential that DBPC trials should include a crossover design in order to better evaluate the response to both gluten and placebo in the same patient.

Another crucial task in the agenda is the identification of diagnostic biomarkers for NCGS. So far, IgG AGA is the only marker positive in about 50% of NCGS patients (5, 38, 55). Further studies are aimed to characterize AGA sub-fractions as specific markers for NCGS. Cytokine / chemokine response measurable at tissue and plasma levels can represent other important markers for NCGS. Preliminary data have shown an increased production of chemokine CXCL10 from the small intestinal mucosa and PBMC in both CD and NCGS as compared to healthy controls [61, 84]. Secretion of CXCL10 is ten-time higher in CD than in NCGS and the entity of the production of this chemokine might be helpful for differentiating CD from NCGS [61]. These data await confirmatory evidence by large series of patients before CXCL10 could be regarded a marker of NCGS.

Although NCGS and CD seem to be two separate entities, the possibility that some cases of NCGS may evolve to CD cannot be disregarded. IgA deposits are regarded as markers of CD identifiable in the small intestinal mucosa before the appearance of serum IgA tTGA [85] and it has been recently reported that a subset of NCGS patients tests positive for IgA deposits to tTG in the small bowel mucosa [86]. Thus, it is advisable to determine IgA deposits to tTG in intestinal biopsies of NCGS patients. On the other hand, Brottveit et al found that among 35 DQ2+ NCGS

patients, only 3 were diagnosed with CD after a gluten challenge including duodenal biopsies and a novel HLA-DQ2-gliadin peptide tetramer test [87]. This test has later been improved and can identify CD patients even without gluten challenge [88]. Ongoing work will elucidate the role of the test in work-up of NCGS patients. Moreover, since NCGS patients display a frequent involvement of skin and nervous system with finding of dermatitis and rash as well as neurological manifestations, it might be noteworthy to detect antibodies to brain TG (TG6) and skin TG (TG3) in NCGS patients [89-91].

Another aspect that should be expanding in coming years is the possible role of intestinal microbiome in generating NCGS. It has been hypothesized that changes in luminal antigens caused by an increased amount of Gram-negative bacteria along with a decrease of Gram-positive Lactobacilli in the intestinal microbiota of mice might trigger the inflammatory response to dietary antigens such as gluten [40]. The possible role of intestinal dysbiosis in the pathogenesis of CD supports further studies in the field of NCGS [41].

Conclusions and summary

NCGS has been recognized as a new gluten-related syndrome by the scientific community, but the knowledge on this syndrome is still incomplete with few certainties and many unsettled issues. The epidemiology of NCGS is to be clarified, but there is growing evidence that it is likely more frequent than CD and mainly affecting young women. NCGS is regarded as an immune-mediated disorder characterized by an activation of innate immunity, but further studies are needed to confirm this and to determine whether adaptive immunity may contribute to its pathogenesis. Besides gluten other wheat proteins, such as ATIs, and FODMAPs might play a role in the development of this syndrome. The diagnosis of NCGS relies on the detection of gastrointestinal (IBS-like) and extra-intestinal symptoms elicited by gluten ingestion and improved by gluten withdrawal in patients with a previous exclusion of CD and WA. DBPC trial is highly

recommended as confirmatory test for NCGS, but so far it has been applied only in research setting and with too many variables, which have limited the reliability of this procedure. The scientific community is searching to improve the diagnostic criteria for NCGS through the following attempts: 1) the definition of a standardized symptom rating scale to objectively assess the improvement of both gastrointestinal and extra-intestinal symptoms after GFD and to define a patient as gluten sensitive; 2) the identification of specific biomarkers, including antibodies to different gluten epitopes and cytokines / chemokines secreted by duodenal mucosa or by PBMC; 3) the standardization of DBPC cross-over trial as a routine test for NCGS diagnosis worldwide.

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Practice points

- NCGS is still a poorly defined syndrome, characterized by gastrointestinal and extra-intestinal symptoms elicited by gluten ingestion in patients without celiac disease and wheat allergy;
- Symptoms disappear quickly in a few hours or days after gluten removal from the diet and recur rapidly when gluten is reintroduced;
- In addition to gluten, other triggers, including ATIs and FODMAPs, can be involved in determining this syndrome;
- NCGS occurs more commonly in young women (3rd-4th decade of life), while it is rare in childhood;
- NCGS prevalence is extremely variable ranging from 0.6% in primary care to 6% in referral centers;
- So far, there are no available biomarker for NCGS, but about 50% of patients are positive for IgG AGA, which disappear quickly after GFD together with symptoms;
- Also, no genetic marker has been identified; in particular, no correlation with HLA-DQ2 and -DQ8, markers of celiac disease, has been demonstrated;
- Double-blind, placebo-controlled, cross-over trial is the best procedure to confirm the diagnosis of NCGS;
- Finally, treatment is based on dietary restriction with particular attention to nutrient intake.

Research agenda

- The pathogenesis of NCGS is not established yet. Whether adaptive concur to innate immune mechanisms remains an open issue awaiting elucidation;
- A standardized rating scale including both gastrointestinal and extra-intestinal symptoms before and after GFD should be adopted for defining a patient as gluten sensitive;
- Research should focus on biomarkers for NCGS (i. e. antibodies to different gluten epitopes and cytokines / chemokines secreted by small intestinal mucosa or by PBMC);
- Based on recent implications, another relevant research task is to characterize the intestinal microbiota in NCGS patients;
- A key objective is the standardization of DBPC crossover trial applicable for NCGS diagnosis worldwide.

Table 1. A proposed standardized symptom-rating scale for identifying NCGS.

	On gluten-containing diet (A)	On gluten-free diet (B)
	Score	Score
Gastrointestinal symptoms		
Abdominal pain	0-3	0-3
Bloating	0-3	0-3
Diarrhea	0-3	0-3
Constipation	0-3	0-3
Alternating bowel	0-3	0-3
Regurgitation (GE reflux)	0-3	0-3
Pyrosis	0-3	0-3
Epigastric pain	0-3	0-3
Nausea	0-3	0-3
Aerophagia	0-3	0-3
Aphthous stomatitis	0-3	0-3
Extraintestinal symptoms		
Tiredness	0-3	0-3
Headache	0-3	0-3
Anxiety	0-3	0-3
Foggy mind	0-3	0-3
Numbness (arms and legs)	0-3	0-3

Joint/muscle pain	0-3	0-3
Skin rash	0-3	0-3
Weight loss	0-3	0-3
Anemia	0-3	0-3
Depression	0-3	0-3
Dermatitis	0-3	0-3
Rhinitis	0-3	0-3
Asthma	0-3	0-3
Total score	X	Y
Decrease of A vs. B score $\geq 50\%$ = high suspicion of NCGS		
Decrease of A vs. B score $< 50\% \geq 25\%$ = low suspicion of NCGS		
Symptoms were scored from 0 to 3 as follows: 0 = absent; 1 = occasionally present; 2 = frequently present; 3 = always present with a severe worsening of quality of life.		

Legend to Figure 1.

Diagnostic flow-chart for patients seen on GFD with a clinical history suggestive of NCGS without a previous exclusion of CD on a gluten-containing diet.

