Journal of the American College of Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uacn20

Non-Celiac Gluten Sensitivity: Literature Review

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Published online: 17 Feb 2014.

To cite this article: Pasquale Mansueto MD , Aurelio Seidita MD , Alberto D'Alcamo MD & Antonio Carroccio MD (2014) Non-Celiac Gluten Sensitivity: Literature Review, Journal of the American College of Nutrition, 33:1, 39-54, DOI: 10.1080/07315724.2014.869996

To link to this article: http://dx.doi.org/10.1080/07315724.2014.869996

Original Research

Non-Celiac Gluten Sensitivity: Literature Review

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Key words: non-celiac gluten sensitivity, celiac disease, wheat allergy, food allergy, HLA, flow cytometric basophil activation test, innate immune response

Background: A significant percentage of the general population report problems caused by wheat and/or gluten ingestion, even though they do not have celiac disease (CD) or wheat allergy (WA), because they test negative both for CD-specific serology and histopathology and for immunoglobulin E (IgE)-mediated assays. Most patients report both gastrointestinal and nongastrointestinal symptoms, and all report improvement of symptoms on a gluten-free diet. This clinical condition has been named non-celiac gluten sensitivity (NCGS).

Aim: We attempt to define the current pathogenic, clinical, and diagnostic criteria of this "new" disease, to provide a practical view that might be useful to evaluate, diagnose, and manage NCGS patients.

Methods: We reviewed the international literature through PubMed and Medline, using the search terms "wheat (hyper)sensitivity," "wheat allergy," "wheat intolerance," "gluten (hyper)sensitivity," and "gluten intolerance," and we discuss current knowledge about NCGS.

Results: It has been demonstrated that patients suffering from NCGS are a heterogeneous group, composed of several subgroups, each characterized by different pathogenesis, clinical history, and, probably, clinical course. NCGS diagnosis can be reached only by excluding CD and WA. Recent evidence shows that a personal history of food allergy in infancy, coexistent atopy, positive for immunoglobulin G (IgG) antigliadin antibodies and flow cytometric basophil activation test, with wheat and duodenal and/or ileum–colon intraepithelial and lamina propria eosinophil counts, could be useful to identify NCGS patients.

Conclusions: Future research should aim to identify reliable biomarkers for NCGS diagnosis and to better define the different NCGS subgroups.

Key teaching points:

- Most patients report both gastrointestinal and nongastrointestinal symptoms, and all agree that there is an improvement of symptoms on a gluten-free diet.
- NCGS diagnosis can be reached only by excluding celiac disease and wheat allergy.
- Patients suffering from NCGS are a heterogeneous group, composed of several subgroups, each characterized by different pathogenesis, clinical history, and, probably, clinical course.
- A personal history of food allergy in infancy, coexistent atopy, positive IgG antigliadin antibodies (AGA) and flow cytometric basophil activation test, with wheat and duodenal and/or ileum–colon intraepithelial and lamina propria eosinophil counts, could be useful to identify NCGS patients.
- Future research should aim to identify reliable biomarkers for NCGS diagnosis and to better define the different NCGS subgroup.

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Abbreviations: CD = celiac disease, WA = wheat allergy, NCGS = non-celiac gluten sensitivity, GFD = gluten-free diet, IBS = irritable bowel syndrome, HLA = human leukocyte antigen, tTg = antitissue transglutaminase, AGA = antigliadin antibodies, EMA = endomysial antigen, DGP = deaminated gliadin peptide, Ig = immunoglobulin, DBPCC = double-blind placebo-controlled challenge, MHC-II = major histocompatibility complex class II, FODMAPs = fructans and other fermentable oligo- and di-monosaccharides and polyols, TCR = T-cell receptor, IELs = intraepithelial lymphocytes, IFN = interferon, IL = interleukin, Treg = T regulation, TGF-β = transforming growth factor-beta, Tr1 = T regulatory type 1, FoxP3 = factor box protein 3, TLRs = toll-like receptors, TJs = tight junctions, OCLN = occludin, CLDNs = claudins, ZO-1 = zonula occludens-1 protein, TJP-1 = tight junction protein 1.

Journal of the American College of Nutrition, Vol. 33, No. 1, 39–54 (2014)
Published by Taylor & Francis Group, LLC

INTRODUCTION AND BACKGROUND

Humankind has existed in some form for about 2.5 million years, but it is only in the last 10,000 years that it has been exposed to wheat. During the 20th century, nutritional needs increased dramatically due to the food shortage cause by the 2 subsequent World Wars in the first half of the century and the exponential growth of the world population in the second half of the century. In this context, the main effort of many agronomists and geneticists was to create new wheat varieties that are stronger and richer in gluten content [1–3]. In 1941, the Nutrition Society, a group of researchers interested in nutritional problems, considered the need to increase wheat production and expand the global wheat output by 5-fold by the end of the 20th century. It is very probable that new kinds of wheat, particularly enriched in gluten content, have greatly contributed to the explosion of gluten-related diseases, although no data in the literature actually confirm this hypothesis. Increased wheat production may have had an effect on celiac disease (CD) prevalence, which was reported in the UK in 1950 as 1:8000 inhabitants and increased to about 1% of the general population in recent decades [4–8].

The recent rise of the market for gluten-free products in the United States, influenced in part by advertising campaigns that claim a medical need for a gluten-free diet (GFD), largely exceeds the foreseeable consumption of the CD patient population. This is just one of the factors that raise questions about possible gluten reactions apart from CD and wheat allergy (WA). A consistent although undefined percentage of the general population consider themselves to be suffering from problems caused by wheat and/or gluten ingestion, even if they do not have CD, because they test negative for CD serology and histopathology. Similarly, these people have no evidence of positive immunoglobulin E (IgE)-mediated assay documenting WA; nevertheless, they exclude wheat and gluten from their diets. In most cases, this happens because of negative experiences reported after eating wheat-containing foods and the benefits derived from a GFD [9–11].

Furthermore, this suggests that the general population has adopted this point of view, relying on self-diagnosis ("hypersensitivity to wheat and gluten") and subsequent therapy (i.e., GFD) far more readily than the medical/scientific community. This can be seen by a 4598:1 ratio of Google to PubMed citations for the keywords "non-celiac gluten sensitivity," as well as several papers expressing clear skepticism or simple caution [12].

The nonmedical specialist press has suggested that "17 million Americans are gluten sensitive." It must be remembered that this is a "big business," with a projected increase in the market for gluten free products from $100 million in 2003, $1.31 billion by 2011, to $1.68 billion by 2015 [13].

Most of these patients report a long clinical history, mainly characterized by gastrointestinal symptoms (abdominal pain and tenderness, irregular bowel habits: constipation or diarrhea or alternating bowel movements); very often they consult a number of physicians, seeking to reach a CD diagnosis but they are considered simply suffering from irritable bowel syndrome (IBS). This unfortunate way of searching for medical help was described by Elena Verdù and colleagues in a clinical review published in 2009 in the *American Journal of Gastroenterology* [14]. This paper had the great value of stimulating the gastroenterology research community to carry out a number of new studies on non-celiac gluten sensitivity (NCGS) and to consider gluten sensitivity as a "fertile crescent" for research [15].

Here we review the international literature about this "new" disease, consulting PubMed and Medline, using the search terms "wheat (hyper)sensitivity," "wheat allergy," "wheat intolerance," "gluten (hyper)sensitivity," and "gluten intolerance;" and we discuss current knowledge about NCGS, seeking to define its current pathogenic, clinical, and diagnostic criteria to provide a practical point of view that might be useful to evaluate, diagnose, and manage NCGS patients.

WHEAT AND GLUTEN: WHAT ARE THEY?

Gluten is the term used to identify the protein mixture of glutelins and gliadins (prolamines), which occurs in the endosperm of wheat and other cereals (such as barley, rye, and spelt) and can be fractionated to produce alpha, beta, and gamma peptides. The ratio of glutelins to gliadins in the protein mixture is approximately 1:1. Gliadins, a group of proteins that are rich in proline and glutamine, have been identified as the main gluten component that is toxic for CD patients [16–18].

Nowadays, gluten is one of the principal dietary components for most of the global population, particularly in Europe and the United States. Mean daily gluten ingestion is 10–20 g in the Mediterranean area and even higher in other populations [19,20].

New variants of wheat have arisen as a result of agricultural mechanization and the growing industrial use of pesticides and fertilizers, which could have a leading role in the adverse immunologic reactions to gluten. Moreover, the process of bread leavening has been progressively shortened, resulting in an increased concentration of toxic gluten peptides in bakery products for all the patients suffering from gluten-related disorders [21,22].

NON-CELIAC GLUTEN SENSITIVITY: A POSSIBLE DEFINITION

Currently NCGS is mainly defined by negative criteria. We talk about NCGS when CD serology is negative, duodenal histology is negative, and IgE-based assays (prick tests or serum-specific IgE dosage) are negative. The only positive requirement to diagnose NCGS is the presence of troubles caused by wheat ingestion and their disappearance on gluten-free/wheat-free diet. In other words, we consider an NCGS diagnosis in all cases that

Table 1. Gluten Sensitivity Diagnosis Criteria

Diagnotics Tools	Celiac Disease	Gluten Sensitivity
Celiac disease serology:		
Antitissue transglutaminase	Positive	Negative
Antigliadins antibodies	Positive	Positive (50% of the cases)
Anti-endomysial antibodies	Positive	Negative
Deaminated gliadin peptide antibodies	Positive	Negative
Duodenal histology (Marsh-Oberhüber classification)	Positive (Marsh 1–3)	Negative (Marsh 0–1)
HLA haplotypes (DQ2-DQ8)	Present	Absent/present
IgE-based assays (prick tests or serum-specific IgE dosage)	Negative	Negative
Clinical features	Troubles caused by wheat ingestion and their disappearance on gluten-free diet	Troubles caused by wheat ingestion and their disappearance on gluten-free/wheat-free diet

HLA = human leukocyte antigen, Ig = immunoglobulins.

lack the key CD criteria (presence of antitissue transglutaminase [anti-tTG] antibodies and endoscopic or histologically significant enteropathy; i.e., Marsh 3) and do not satisfy the criteria for IgE-mediated wheat allergy but respond to gluten exclusion (Table 1) [9,10,23–27]. NCGS is frequently perceived by the patients themselves and they consult physicians seeking to reach a definite diagnosis of CD, or at least of "wheat hypersensitivity," but as aforesaid this is generally opposed, because the patients do not fulfill CD diagnostic criteria and do not show laboratory assays documenting an IgE-mediated food allergy. On the other hand, the role of emotion is known to be pivotal in these patients. Consequently, a clinical response to elimination diet and the use of double-blind placebo-controlled challenge (DBPCC) to confirm NCGS diagnosis are recommended, even though DBPCC is quite cumbersome and time consuming and therefore very rarely employed. For many years, these patients continued to consume gluten-containing foods because gluten was not considered to be the cause of their symptoms. As a result, they were left in a no man's land, unrecognized by either allergists or gastroenterologists. Much like patients who had IBS, NCGS patients were commonly referred to psychiatrists because they were believed to have an underlying mental illness as a result of the poor physician awareness of this disease [9,23–27].

In 2011 an international panel of experts from 14 countries participated in a consensus meeting in London and defined NCGS as "a non-allergic and non-autoimmune condition in which the consumption of gluten can lead to symptoms similar to those seen in CD." The consensus statement suggested that clinical symptoms can overlap with CD or WA, respond to GFD, and worsen on gluten reintroduction, but patients must be characterized by negative CD-specific antibodies (absence of anti-tTG antibodies, anti-endomysial antibodies [EMA], or deaminated gliadin peptide [DGP] antibodies) and normal duodenal histology, even if an increased density of CD3+ IELs could be detected, as reported by Sapone et al. [23]. In summary, the hallmarks for NCGS diagnosis are clinical improvement on a GFD in the absence of anti-tTG, EMA, or DGP antibodies and intestinal mucosal abnormalities [23].

The concept of NCGS has challenged physicians and investigators over the years. Data published in 1980 and 2000 suggested the actual existence of a syndrome caused by the ingestion of gluten in a subset of patients who did not have CD or WA, even though it has been suggested that some of these patients might be affected from "potential CD" [28,29].

However, most published descriptions of this potential disease involve patients with positive serology, associated with intraepithelial lymphocytosis in the duodenum (Marsh-Oberhüber modified classification 0–1). In other words, they may just be CD patients not fulfilling the classic diagnostic criteria. Therefore, we had to check the existing literature to carefully select available works, considering only those closely meeting the above-mentioned NCGS criteria [27,30].

PATHOGENESIS OF CD, WA, AND NCGS

The role of gluten in CD is clear. Toxic peptide sequences have been determined, genetic susceptibility loci have been identified, and pathological processes are fairly well known. Deamidation of gliadin epitopes by tTG enables them to be presented with a high affinity, in genetically susceptible individuals, to major histocompatibility complex class II (MHC-II) T-cells. This process initiates a series of events, resulting in mucosal inflammation, small intestinal villous atrophy, increased intestinal permeability, macro- and micronutrient malabsorption, and resultant CD complications. The disease is an autoimmune disorder, as heralded by the demonstration of specific serologic markers, most notably serum anti-tTG antibodies, autoimmune enteropathy, and autoimmune comorbidities (e.g., autoimmune thyroiditis, type-1 diabetes, etc.) [31–33].

In WA, immunoglobulin E (IgE), cross-linking by repeated sequences in gluten peptides (for example, Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe), triggers the release of chemical mediators, such as histamine, from basophils and mast cells [34,35].

On the contrary, NCGS pathogenesis is still largely undetermined. A preliminary key issue is whether symptoms are

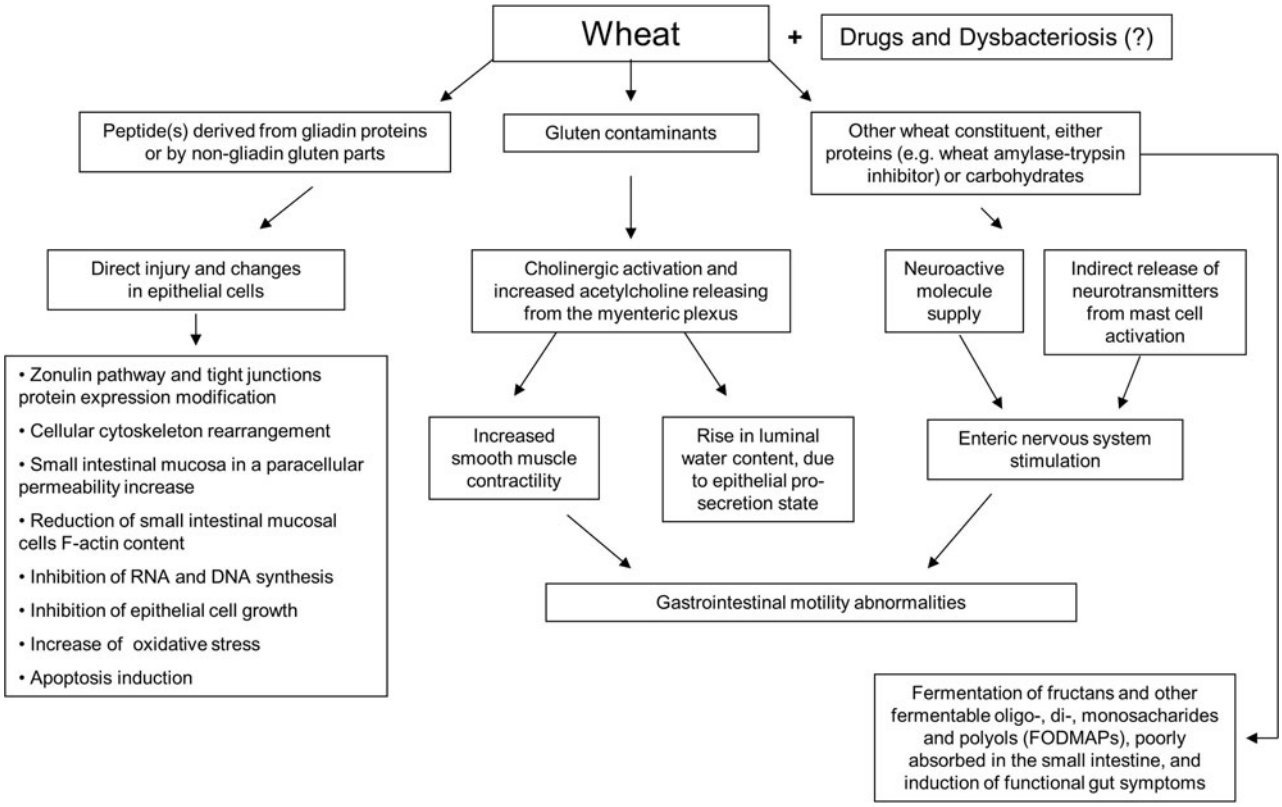

Fig. 1. Damage and abnormalities in epithelial cells induced by wheat through nonimmunomediated mechanisms.

being induced by peptide(s) derived from gliadin proteins or by nongliadin gluten parts, or by gluten contaminants, or by other wheat constituents, such as more protein (e.g., wheat amylase–trypsin inhibitor) or carbohydrates. Broad *in vitro* evidence, both in celiac and non-celiac experimental models, points out how gluten and gliadin might directly cause damage and abnormalities in epithelial cells by nonimmunomediated mechanisms (Fig. 1). For example, gliadin is known to cause cellular cytoskeleton rearrangement, through the zonulin pathway, and loss of tight junctions, modifying protein expression, which results in a paracellular permeability increase of the small intestine mucosa [36–41]. Moreover, gliadin has a toxic effect because it reduces small intestinal mucosal cell F-actin content, inhibits RNA and DNA synthesis, inhibits epithelial cell growth, increases oxidative stress, and induces apoptosis, thereby altering mucosal homeostasis [42–46]. Alternatively, gluten might cause gastrointestinal neuromuscular abnormalities by increased acetylcholine release from the myenteric plexus and consequent cholinergic activation, as indicated by experimental models in DQ8-restricted mice. This might lead to an increase in smooth muscle contractility, and indirectly luminal water content rise, due to epithelial prosecretory state, a neuromediated effect. Clearly, other wheat antigens may act in a similar way, too. Symptoms might also be induced by enteric nervous system stimulation both directly, by neuroactive molecule supply, and by indirect release of neurotransmitters from, for example, mast cell activation. Neural active peptides from gluten or wheat ingestion might potentially gain access to enteric nerve endings, but these are still unknown and their absorption might seem less likely, given normal intestinal permeability in NCGS patients (see section labeled "Intestinal Mucosa Epithelial Barrier Function"). Newer techniques, such as examining basophil activation in response to gluten or wheat stimulation, might suggest other pathological mechanisms for gluten-related symptoms [47–49]. In accordance with the above-mentioned gluten effect on gastrointestinal neuromuscular function in experimental models, it is quite frequent in clinical practice to examine CD patients showing gastrointestinal motor abnormalities, similar to those of IBS. In fact, in 30%–60% of patients, physical examination and dyspeptic/dysmotility symptoms (epigastric discomfort, early satiety, etc.) suggest a gastrointestinal motility disorder [50]. Consistent data are now available on the presence of disturbed motility of the esophagus, stomach, small intestine, gallbladder, and colon of untreated celiac patients. However, gastrointestinal abnormalities differ in various gastrointestinal locations: esophageal transit, gastric and gallbladder emptying, and orocecal transit time are delayed, and small bowel and colonic transit is faster. Motility disorders of the gut in CD patients are also a predisposing factor in the development of small intestinal bacterial overgrowth and may contribute both to the development of symptoms in some untreated CD patients and to a persistence of symptoms after gluten-free diet in some other CD

patients. Therefore, surveillance for CD in patients complaining of dysmotility-like dyspeptic symptoms or IBS should be increased [50–57]. In this context, it has been demonstrated that small bowel and/or colonic transit is speeded up in 46% of patients with diarrhea-predominant IBS. Improvement in these patients with gluten withdrawal is associated with HLA-DQ2/DQ8 positivity. Patients positive for HLA-DQ2 and HLA-DQ8 had faster small bowel and/or colonic transit compared to HLA-DQ2-negative and HLA-DQ8-negative patients. In contrast, gastric emptying seems not to be associated with HLA-DQ2 and HLA-DQ8 status [58]. However, HLA-DQ2/DQ8 expression in these patients might be markers of potential CD in a subgroup of IBS patients, who consequently appear to take advantages of a gluten-free diet [59]. Therefore, it is possible to speculate that gluten-/gliadin-induced gastrointestinal motility abnormalities might also be involved in NCGS patients, as demonstrated in experimental models and in CD patients, and be responsible, at least in part, for some of NCGS patient symptoms. Carbohydrates, among nongliadin and nongluten components of wheat, may also be considered a likely candidate, especially fructans and other fermentable oligo- and di-monosaccharides and polyols, because they are poorly absorbed in the small intestine and may induce fermentation and functional gut symptoms [60]. Therefore, symptom induction might be a wheat-specific rather than a gluten-specific phenomenon, so the term NCGS should possibly be replaced by *wheat sensitivity*. Finally, other luminal antigens, such as drugs or intestinal microbial components (dysbacteriosis), might contribute to enhanced inflammatory responses to dietary antigens such as gluten or wheat [61,62].

THE TWO SIDES OF NCGS IMMUNOPATHOLOGY AND INTESTINAL BARRIER FUNCTION

Researchers evaluated NCGS pathology considering 2 different sides of the problem: the possible role of innate *versus* adaptive immunity and the intestinal mucosa epithelial barrier function (Table 2 and Fig. 2).

Innate Versus Adaptive Immunity

Acquired data indicate that NCGS is not related to the genetic pattern found in most patients with CD. Though CD is characterized by a strong genetic association with the MHC-II haplotype (about 95% of patients carrying HLA-DQ2, and the remaining 5% carrying HLA-DQ8), only about 50% of patients with NCGS carry HLA-DQ2 and/or HLA-DQ8, a percentage slightly higher than the general population (30%); all of these data suggest a reduced involvement of MHC-dependent adaptive immune response in NCGS compared to CD. In addition, serology for common CD auto-antibodies (e.g., anti-tTG) is negative [63–65]. NCGS mucosa contain increased numbers of CD3+ T-cells and T-cell receptor-alpha/beta intraepithelial lymphocytes (IELs) compared to controls but lower than those in active CD

Table 2. Celiac Disease, Gluten Sensitivity, and Food Allergy Immunologic and Intestinal Epithelial Barrier Function Findings

Immunologic and Intestinal Epithelial Barrier Features	Celiac Disease	Gluten Sensitivity	Food Allergy
HLA-DQ2/DQ8	Present	Present (lower than CD)/absent	Present/absent
CD3+ intraepithelial lymphocytes	Increased	Increased (lower than CD)	Normal
TCR-alpha/beta IELs	Increased	Increased (lower than CD)	Normal
TCR-gamma/delta IELs	Increased	Normal	Increased
T_H1 and T_H17 clones expansion and cytokines production (e.g., IFN-gamma, IL-6, IL-17A, and IL-21)	Increased	Normal	Increased (in addition to T_H2 clone expansion and cytokines production)
T regulation (T_{reg}) clones expansion and cytokines/messengers production (e.g., FoxP3, TGF-beta 1, TGF-β1, and IL-10)	Increased/reduced	Reduced	Increased/reduced
TLRs expression	Increased TLR4 and TLR9 expression (but not of TLR3 and TLR7), and reduced TLR2 expression	Increased TLR2 expression and to a lesser extent of TLR1 (but not of TLR4)	Reduced TLR2 and TLR4 expression
Intestinal mucosal permeability (assessed by lactulose–mannitol test)	Increased	Reduced	Increased
Tight junction protein expressions:			
CLDN4	Normal	Increased	NA
CLDN1	Normal	Normal	Increased
CLDN2	Normal	Normal	NA
OCLN	Normal	Normal	Increased
TJP-1	Normal	Normal	Increased

CD = celiac disease, TCR = T-cell receptor, IEL = intraepithelial lymphocytes, IFN = interferon, IL = interleukin, TGF = transforming growth factor, TLR = toll-like receptor, HLA = human leukocyte antigen, CLDN = claudin, OCLN = occludin, TJP-1 = tight junction protein 1.

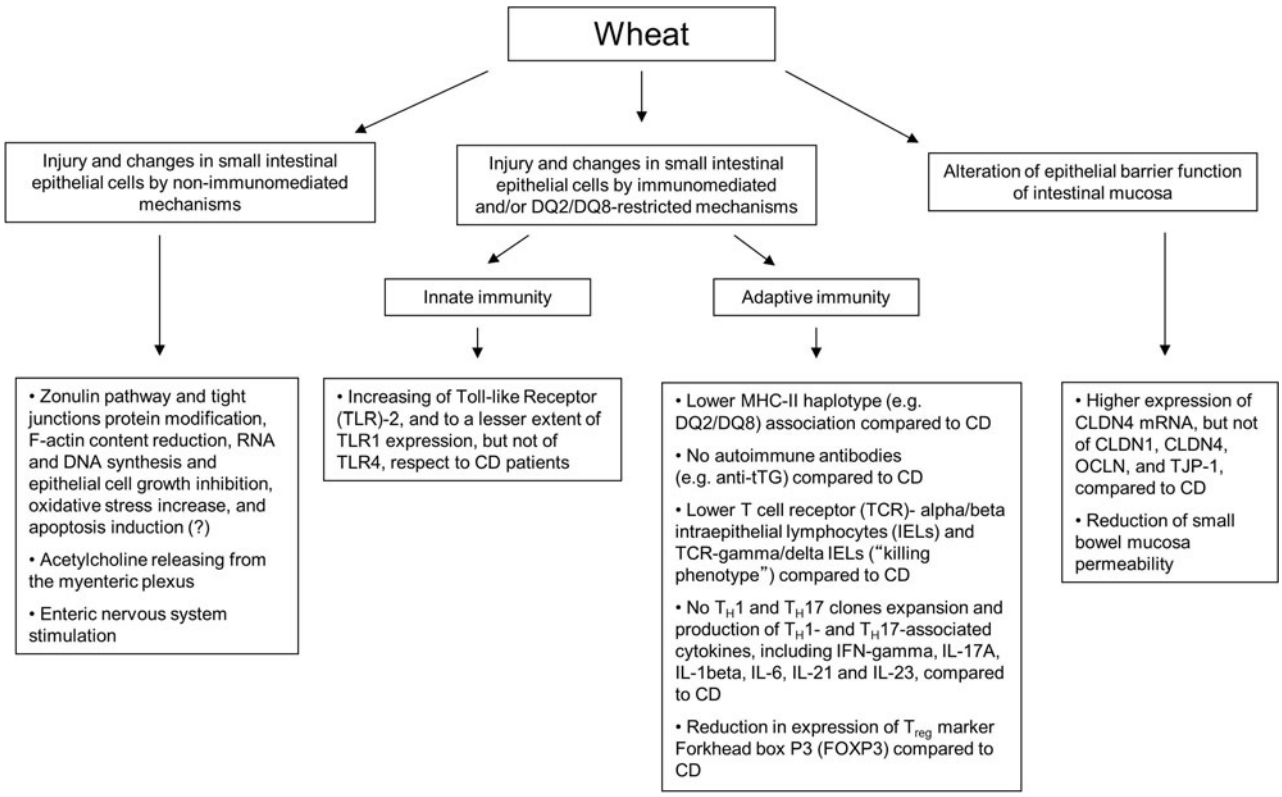

Fig. 2. Innate and adaptive immunity response to wheat.

patients and in the context of a relatively conserved villous architecture (0 and 1 stages of the Marsh-Oberhüber modified classification). Numbers of T-cell receptor-gamma/delta IELs (killing phenotype) were elevated only in CD patients, whereas NCGS patients were similar to controls. This is consistent with a more limited involvement of the adaptive immune system in NCGS compared to CD and may also explain why this condition seems not to be accompanied by significant autoimmune phenomena [63,64].

CD has been considered a classical T_H1-mediated disorder because of the enhanced mucosal mRNA expression of interferon (IFN)-gamma, but not of interleukin (IL)-4, in patients with untreated disease. Following the identification of the T_H17 T cell subset (interleukin [IL]-17-producing CD4+ T helper cells), and the growing evidence that these cells are centrally involved in the pathogenesis of autoimmune disorders, such as collagen-induced arthritis and colitis, it has become important to investigate the possible involvement of T_H17 cells in CD. T_H17-associated cytokines expression—for example, IL-17A—is higher in patients with active CD as opposed to patients on a GFD. In addition, it has been demonstrated that gliadin can induce T_H17-polarizing cytokines—for example, IL-1beta and IL-23 production in peripheral blood monocytes—providing a possible causative link between gluten exposure and T_H17 cell expansion in CD. Unlike NCGS patients, CD patients show increased levels of adaptive immune markers in the small intestinal mucosa, triggered by DQ2 DQ8-bounded tTG-deamidated gluten peptides and associated with T_H1 and T_H17 clone activation and T_H1- and T_H17-associated cytokine production, including IFN-gamma, IL-17A, IL-6 (pleiotropic cytokine promoting differentiation and function of T_H17 cells), and IL-21 (also related to T_H1 and T_H17 cell pathology) [63–69].

Another interesting immunological finding that might distinguish NCGS from CD concerns mucosal expression of genes associated with T-regulation (T_{reg}) cells. In CD and CD-related autoimmune diseases a resistance of effector T lymphocytes to suppression by adaptive T_{reg} cells has been demonstrated and has been proposed to explain the loss of immune homeostasis and development of autoimmune responses. Although percentages of CD4+ CD25+ FoxP3+ intraepithelial and lamina propria lymphocytes were significantly higher in patients with active CD compared to healthy controls, proliferation and IFN-γ production of intestinal T lymphocytes were significantly less inhibited by autologous or heterologous T_{reg} cells in CD patients than in controls [70]. Several T_{reg} cells have been found to be important for oral food tolerance: T_H3 cells, a population of CD4+ cells that produce transforming growth factor–beta (TGF-β); T regulatory type 1 (Tr1) cells, which secrete IL-10; CD4+ CD25+ regulatory T-cells, which express the transcription factor forkhead/winged-helix transcription factor box protein 3 (FoxP3); CD8+ suppressor T-cells; and gamma-delta T-cells. Natural T_{reg} cells are CD4+ CD25+ T-cells that develop and migrate from the thymus to perform their key role in immune homeostasis, whereas adaptive T_{reg} cells are

nonregulatory CD4+ T-cells that acquire CD25 (IL-2R alpha) expression outside of the thymus and are typically induced by inflammation and disease processes, such as autoimmunity and cancer [71]. Considering the lack of association between NCGS and autoimmune serology and/or diseases, it is possible to assume that adaptive T_{reg} cells efficiently prevent progression to this kind of response in NCGS patients. Surprisingly, expression of FoxP3 was found to be reduced in patients with NCGS compared to CD, perhaps in the context of a generally reduced activation of adaptive immunity in NCGS relative to CD patients [63]. However, the overall expression of this and other messengers (e.g., TGF-β1, and IL-10) represents a rather controversial aspect of CD autoimmunity, because both downregulation and upregulation of FoxP3 and other T_{reg}-dependent molecules have been reported in patients with CD and related autoimmune conditions (e.g., type 1 diabetes mellitus) [72–76].

Among innate immune mechanisms, toll-like receptors (TLRs) represent a family of evolutionarily conserved receptors able to detect microbial invasion via pattern recognition and to mediate a rapid inflammatory response, inducing type I interferon and other cytokines, which may or may not progress into an antigen-dependent adaptive response. The expression of TLR2 (and to a lesser extent TLR1 but not TLR4) is considerably higher in the intestinal mucosa of NCGS patients compared to CD patients, confirming a prevalent role of the innate immune system in the pathogenesis of NCGS [63]. However, innate immunity and TLR expression and function are also important in CD pathogenesis. TLR4 expression levels (but not TLR3 and TLR7) on duodenal biopsies are higher in CD specimens compared to controls. CD patients with high TLR4 levels also show high levels of interleukins (IL-1, IL-6, IL-8, and IL-17) as well as transcription factors (IRAK4, MyD88, and NF-κB) [77,78]. In another study, TLR2 mRNA expression was significantly lower in untreated and treated celiac patients, whereas TLR9 mRNA expression was higher in untreated celiac patients compared to controls [79].

Taken together, these preliminary data seem to suggest that innate rather than adaptive immunity has a prominent pathogenic role in NCGS. The main involvement of innate versus adaptive immune pathways might help explain the clinical and serological differences that can be seen in CD versus NCGS patients. However, it must be considered that there are only a few studies available, mainly on experimental models, with a possible bias in NCGS patient selection (e.g., overlap with CD), due to the lack of clearly defined NCGS diagnostic criteria. Therefore, other pathogenic mechanisms might be supposed; for example, not IgE-mediated allergic mechanisms (see section labeled "Non-Celiac Gluten Sensitivity or Gluten Allergy?").

Intestinal Mucosa Epithelial Barrier Function

Small intestine mucosa epithelial barrier modifications represent another possible factor involved in the pathogenesis of gluten-related disorders; that is, CD and NCGS. Loss of intestinal barrier function, which has been clearly established in CD, represents a key mechanism for autoimmunity development, by the continuous and aberrant passage of antigens through intestinal epithelium. This may cause an immunity switch from tolerance to response, hence representing an increased risk for autoimmune diseases in subjects with particular genetic determinants, both MHC and non-MHC, conditioning inappropriate antigen processing and presentation [80–82]. However, patients with NCGS, unlike CD, did not show changes (or show reduction) in intestinal mucosa permeability, as assessed by the lactulose–mannitol test. In particular, Sapone et al. demonstrated an increased lactulose-to-mannitol urinary ratio, indicating enhanced permeability of the small intestine, in patients with CD but not in NCGS [63]. In addition, Biesiekierski et al., using a dual sugar absorption test (i.e., cellobiose/mannitol sugar permeability test), did not find any significant difference in the intestinal barrier function of 2 randomly treated groups of NCGS patients, one challenged by gluten and the other by placebo [83].

In the intestinal epithelium, paracellular permeability is regulated by intercellular tight junctions (TJs) and multiple proteins forming cTJs strands (e.g., occludin [OCLN], claudins [CLDNs], and zonula occludens-1 protein, also known as tight junction protein 1 [TJP-1]). TJs have a critical role in the development of intestinal immunological responses. When TJs' integrity is compromised, an immune response to environmental antigens, which might cross-react with host antigens, may develop, thereby triggering CD onset [40,84,85]. In particular, CLDNs are integral TJ components, critical for maintaining cell–cell adhesion in epithelial monolayers. The overall balance of CLDN species, expressed in a specific cell type, determine the permeability of its TJs. For instance, CLDN1 and CLDN4 are postulated to decrease TJ-dependent permeability, whereas CLDN2 is postulated to increase TJ-dependent permeability [86]. As recently pointed out, in duodenal biopsy samples from NCGS patients, TJs component polymerase chain reaction analysis showed a notably higher expression of CLDN4 mRNA compared to CD patients. This finding suggests that the former might have a less permeable mucosa than the latter. Other CLDN genes (e.g., CLDN1 and CLDN2) as well as other genes associated with TJ function (i.e., OCLN and TJP-1) did not appear to be expressed differently in NCGS and CD mucosa [63].

NON-CELIAC GLUTEN SENSITIVITY OR GLUTEN ALLERGY?

In a recent study, we demonstrated that a high-frequency characteristic of NCGS patients is the coexistence of multiple food intolerance, including cow's milk, egg, and other foods. In this way, we suggested the existence of at least 2 distinct groups of NCGS patients: the first characterized by NCGS patients alone and the other by patients intolerant to wheat, cow's milk

protein, and many other foods (multiple foods hypersensitivity, including NCGS). Patients showing multiple food hypersensitivity presented characteristics more similar to allergy rather than to CD, although none of them tested positive for IgE-based assays. In accordance with an allergy hypothesis, these patients showed a higher frequency of family and personal history of food allergy, especially in infancy, and coexistent atopy than the other group. Their predominant presence in the study group probably conditioned the results of the immunology assays (basophil activation assay, IgG antigliadin antibodies [AGA] and anti-betalactoglobulin positivity) and of the histology studies (mucosal eosinophil infiltration in the duodenum and colon). Therefore, it is possible to hypothesize an allergic, not IgE-mediated, pathogenesis in the development and presentation of gluten sensitivity abnormalities and symptoms [87].

Available data do not seem to support this point of view, but they refer especially to IgE-mediated experimental patient surveys. Patients with untreated food allergy express equal densities of total intraepithelial CD3+ and alpha/beta + T-cells but significantly higher densities of gamma/delta + cells and gamma/delta + /CD3+ ratio than patients currently on an elimination diet for food allergy or healthy controls [88–90].

In the context of food allergy, in contrast to NCGS, where no T_H1 and/or T_H17 clone expansion and/or cytokine production have been demonstrated, it has been shown, especially in experimental models (mice), that not only T_H2 cytokines (IL-5, IL-13, and IL-10) but also T_H1 cytokines—for example, IFN-gamma—and T_H17 cytokines—for example, IL-17—are produced. Interestingly, T_H17 cells and their associated cytokines (the IL-17 family, IL-21, IL-22, and IL-23) have paradoxical effects at the intestinal level, because they display both protective/homeostatic and pathogenic/inflammatory functions. However, functional studies provided evidence for the different roles of IL-17A and IL-17E in the regulation of immune responses: IL-17A is involved in inflammation, and IL-17E is able to induce T_H2 cytokine production and eosinophilia. Therefore, IL-17E but not IL-17A is associated with allergic sensitization [91–94].

Similarly to CD, data about FoxP3 expression and food allergy are quite contradictory. Like NCGS patients, children with food allergy show statistically significant lower levels of the FoxP3 and IL-10 gene expression than healthy children. Those acquiring tolerance to the food show significantly higher levels of the FoxP3 gene expression than children with active food allergy [95]. However, whereas some studies showed normal FoxP3 levels in infants with milk and egg allergy, some others demonstrated FoxP3 + cells increasing in the duodenum of patients with untreated food allergy, even if these cells are not able to suppress the harmful immune response, indicated by the low FoxP3 transcript expression [96–98].

Moreover, other studies showed that food allergy mechanisms are more similar to those of CD than those reported in NCGS. In contrast to NCGS, but similar to CD, recent studies indicated that TLR polymorphisms or their impaired signaling, specifically TLR-2 and TLR-4, were correlated with a higher risk for food allergy, through effects on innate immune pathways, even if other ones did not demonstrate such a correlation. TLR2- and TLR4-dependent signals, provided by the intestinal commensal flora, inhibit T_H2-mediated allergic response development to food antigens, by antigenic stimulation of the gut associated lymphoid tissue (GALT), modification of lymphocyte responsiveness, and generation of T_H1-based memory effectors [96,99–101].

In contrast to NCGS, but similarly to CD, impaired intestinal permeability, evaluated by lactulose–mannitol ratio urinary detection, can also be detected in patients with adverse reactions to food, including food allergy. A statistically significant association has been demonstrated between the severity of referred clinical symptoms and the increase in the intestinal permeability index [102,103]. In patients with cow's milk allergy or intolerance, cellobiose/mannitol sugar permeability test, performed before and after cow's milk challenge, showed alteration of intestinal permeability induced by milk, suggesting the usefulness of the sugar permeability test, in addition to clinical observation, as an aid in the evaluation of challenge tests in infants with suspected cow's milk allergy or intolerance [104,105].

A relationship between intestinal TJ protein expression and food allergy has been evaluated in a single study, demonstrating that exposure of small intestinal biopsy specimens of patients with food allergy to food allergens led to a significant increase in IgE-positive cells with an enhanced histamine and tryptase release and an altered expression of tight junction proteins—for example, CLDN1, OCLN, and TJP-1—in contrast to NCGS, where they are normally expressed. To date, no data are available regarding other CLDNs; for example, CLDN2 and CLDN4 [106].

CLINICAL CHARACTERISTICS OF NCGS

NCGS epidemiology is far from established. NCGS prevalence was reported to be about 6% based on the Maryland clinic experience (where between 2004 and 2010, 5896 patients were evaluated and 347 fulfilled NCGS diagnostic criteria), even though these data could overestimate the real prevalence of the disease, having been recorded in a referral center [23,64]. Furthermore, a recent paper, summarizing the results from the continuous National Health and Nutrition Examination Survey (years 2009–2010), reported a possible prevalence of NCGS of 0.55% in the general population in the United States [107]. It is possible that the real prevalence of NCGS is intermediate in this range (0.55%–6%).

It has been reported that NCGS onset is at a median age of 40 years; however, our recent study, including the largest series of NCGS patients, showed a median age of 28 years, thus suggesting that NCGS affects patients younger than previously reported, and functional bowel disorders (including IBS) are

more prevalent in females than in males (male-to-female ratio ranging between 1:2.5 and 1:4) [25,87].

NCGS is clinically characterized by symptoms/signs that usually occur soon after gluten ingestion, improving or disappearing (within hours or a few days) with gluten withdrawal and relapsing following its reintroduction. Clinical presentation of NCGS is a combination of IBS-like symptoms (e.g., bloating, abdominal pain, bowel habit abnormalities [either diarrhea and/or constipation]), and systemic manifestations (e.g., foggy mind, headache, fatigue, depression, joint and muscle pain, leg or arm numbness, dermatitis [eczema or skin rash], and anemia) [9,10,23–25,87]. Biesiekierski et al., in a double-blind randomized placebo-controlled trial, conducted on patients with IBS in whom CD was excluded and who were symptomatically controlled on a GFD, proved that IBS-like symptoms and tiredness reoccurred more frequently in the gluten-challenged group than in the placebo group (68% and 40%, respectively), thus suggesting a link between gluten assumption and symptom origin [83]. Although the frequency of intestinal IBS-like symptoms is higher than extraintestinal manifestations, all patients usually report 2 or more extraintestinal symptoms, the most common being foggy mind (42%), defined as a sensation of lethargy that occurs after eating gluten-containing foods, and fatigue (36%) [65]. However, the authors reported that only one extraintestinal manifestation (tiredness) was associated with IBS-like symptoms [83]. Thus, other data are needed to establish the actual prevalence and type of extraintestinal symptoms in NCGS patients.

Brottveit et al.[108] considered the presence of somatization, personality traits, anxiety, depression, and health-related quality of life in NCGS patients compared to CD patients and healthy controls and compared the response to gluten challenge in the former and the latter. NCGS patients did not exhibit any tendency for general somatization. Personality and quality of life did not differ between NCGS and CD patients and were mostly at the same level as in healthy controls. NCGS patients reported more abdominal and nonabdominal symptoms than CD patients after gluten challenge [109].

Unlike CD patients, NCGS patients do not seem to have autoimmune comorbidities. In a group of 78 NCGS patients, none had type 1 diabetes mellitus and only one (1.3%) had autoimmune thyroiditis, compared to 5% and 19%, respectively, of 80 CD patients [65].

Recently we retrospectively reviewed the characteristics of a large group of IBS-like patients, fulfilling the newly proposed NCGS criteria, and showed that a considerable percentage (one fourth) of these patients who underwent DBPCC wheat challenge, were actually suffering from NCGS. The study showed how food allergy history in infancy, coexistent atopic diseases, weight loss, and anemia were more frequent in NCGS patients than in IBS controls. We suggested that weight loss and anemia might be due, at least in part, to the self-restricted diet started by the patients, which usually excluded many foods. A high-frequency NCGS patient characteristic was the coexistence of multiple food intolerance, including cow's milk, egg, and other foods. In this way, we suggested the existence of at least 2 distinct groups of NCGS patients (Fig. 3): one characterized by NCGS alone and the other by patients intolerant to wheat, cow's milk protein, and many other foods (multiple food hypersensitivity, including NCGS). Patients belonging to the first group showed a higher frequency of HLA-DQ2 or -DQ8 haplotype; furthermore, duodenal lymphocytosis was shown in 94% of cases, and EMA assay in the culture medium of duodenal biopsies tested positive in one third of them. Considering that symptomatic patients, who produce EMA in the duodenal culture, can subsequently develop villous atrophy when remaining on a gluten-containing diet, it is possible to hypothesize that a percentage of these patients could be predisposed to develop villous atrophy in the future. The second group (multiple food hypersensitivity, including NCGS) presented with characteristics more similar to allergy rather than CD patients, although none of them tested positive for IgE-based assays. These patients showed a higher frequency of personal history of food allergy in infancy and coexistent atopy than the other group [87,110,111].

DIAGNOSTIC APPROACH TO NCGS

Although CD and NCGS seem to be 2 different conditions, it has been reported that 10 of 78 (12.8%) patients with NCGS were first-degree relatives of CD patients [65]. Furthermore, it is known that local in-rectum gluten instillation can be a useful test to identify mucosal evidence of NCGS at an early stage in asymptomatic first-degree relatives of CD patients [112,113]. Both of the above-mentioned characteristics could be a link between NCGS and CD and could lead us to hypothesize that a percentage of NCGS patients could represent the first initial stage of CD. Consequently, the first step in evaluating patients suffering from suspected NCGS is to distinguish between this condition and a very early CD stage. In this regard, we suggest the usefulness of the HLA haplotype determination to search for the DQ2 or DQ8 forming alleles. In fact, due to the high negative predictive value of the genetic assay, in the patients who will result negative for DQ2 and DQ8 haplotype, the CD diagnosis can be excluded. For the patients carrying the DQ2 or the DQ8 haplotypes, a successive useful approach is the anti-endomysium assay (EmA assay) in the culture medium of duodenal biopsies [110,111]. In a group of NCGS patients we found subjects with negative CD serology, normal small intestinal mucosa, but positive detection of EMA in the culture medium of duodenal biopsies [87]. Because a direct correlation between the serum EMA titer and the severity of the intestinal histology damage has been demonstrated [114], it is very probable that this subgroup of NCGS patients really consisted of CD subjects in whom positive serology and intestinal villi atrophy will develop in the

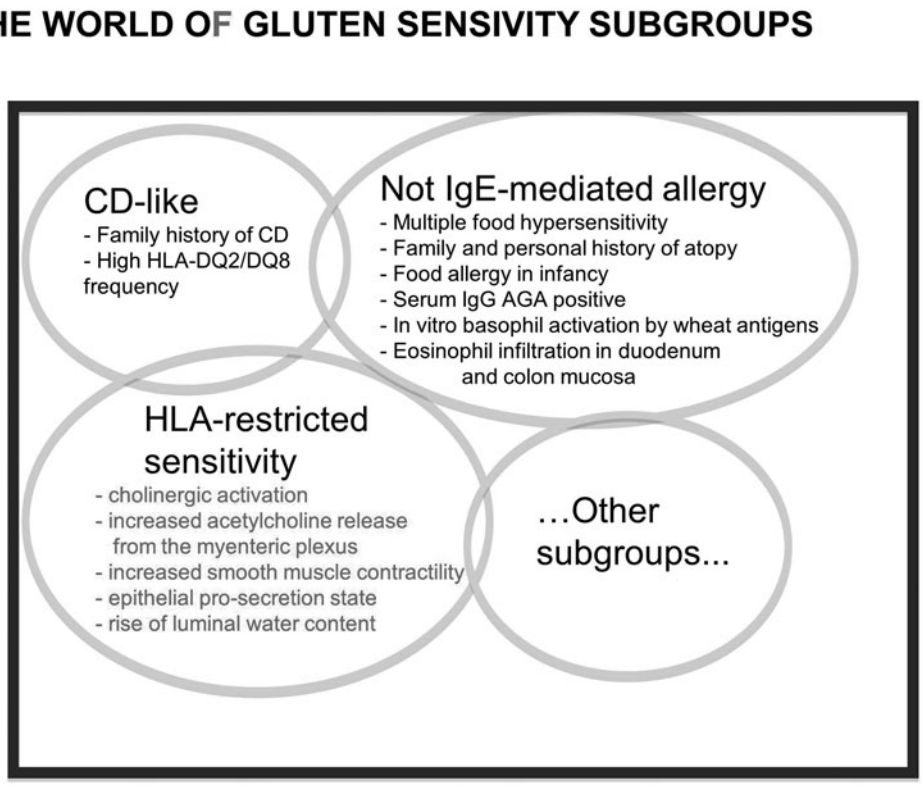

Fig. 3. Distinct groups of NCGS patients.

future [110,111]. A similar progression has been demonstrated in symptomatic patients showing positive serum EmA and normal villi architecture [115].

Elimination diet and open challenge (i.e., monitored reintroduction of gluten-containing foods) are often used to evaluate whether the patient's health improves with the elimination or reduction of gluten from the diet and relapses after gluten reintroduction. Gluten withdrawal is associated with a dramatic improvement or even the disappearance of IBS-like and extraintestinal symptoms, and reintroducing gluten causes symptom recurrence. Symptom discontinuance or reoccurrence, attributable to the absence or presence of dietary gluten, should be considered a test indicating NCGS [9,10,23–25]. However, as aforesaid, because a placebo effect produced by gluten withdrawal cannot be excluded, DBPCC studies are appropriate to confirm NCGS diagnosis [87].

NCGS patients frequently report a personal history of food allergy in infancy and coexistent atopy, so it is mandatory for physicians to enquire about these topics. These characteristics are more frequent in patients with multiple food hypersensitivity. This subgroup of NCGS patients probably has a high frequency of positive immunologic assays. In fact, about half of NCGS patients had positive first-generation AGAs, especially of the IgG class [87]. Although lower than in CD patients (80%–90%), prevalence of IgG AGA in patients with NCGS is much higher than in those with a variety of other gastrointestinal—for example, IBS (20%) [116]—or nongastrointestinal diseases (e.g., connective tissue disorders and autoimmune liver disease, 9% and 21.5%, respectively) or in the general population and healthy blood donors (ranging from 2% to 8%) [117–122]. Therefore, in the presence of clinical symptoms that suggest NCGS, IgG AGA positivity, together with negative anti-tTG, EMA, and DGP antibodies, NCGS diagnosis might be suspected.

More interestingly, an *in vitro* flow cytometric basophil activation test with wheat (surface CD-63 expression) confirmed a high sensitivity for NCGS diagnosis and seems to be, to date, the most accurate NCGS marker [87,123].

Colon histology evaluation showed intraepithelial and lamina propria eosinophil infiltration in about two thirds of cases; this last finding was also frequently observed in the duodenum, together with lymphocytosis. The diffuse ileum–colon involvement could explain why the main symptoms in these patients were lower (i.e., IBS-like ones) and not upper (i.e., dyspepsia-like one) ones and define a histology pattern pointing to a NCGS diagnosis [87].

Due to the lack of diagnostic tests for this condition, diagnosis is essentially made by exclusion (especially of CD and wheat allergy). An intestinal biopsy sample should always be obtained from patients with suspected NCGS when they are on a gluten-containing diet to exclude the presence of villous atrophy, the hallmark of CD histopathology. About 60% of NCGS patients have normal intestinal mucosa, with <25% IELs (grade

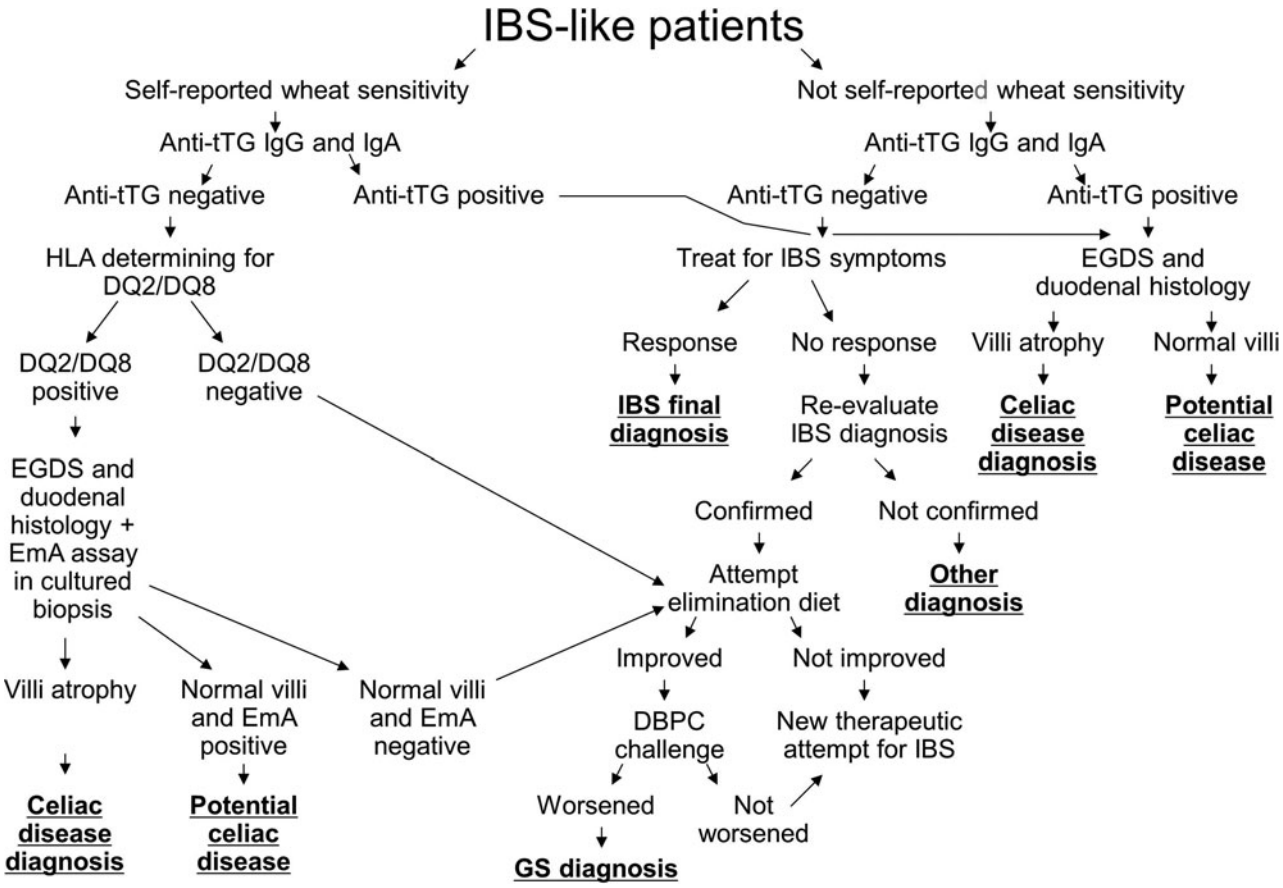

Fig. 4. Diagnostic flowchart proposed for patients with suspected NCGS or CD.

Table 3. Future Research Field Throughout Gluten Sensitivity Hot Topics

Topic	Possible Research Field
Pathogenetic role of peptide(s) derived from gliadin proteins or by nongliadin gluten parts, or of gluten contaminants, or other wheat constituent, either proteins (e.g., wheat amylase–trypsin inhibitor) or carbohydrates	Double-blind placebo-controlled studies testing whole wheat in comparison to its single component (gliadin, glutenin, gluten contaminants, others wheat component) to asses actual patient intolerance (gluten sensitivity or wheat sensitivity?)
Innate or adaptive immunologic mechanisms	Evaluation of cytokines pattern in colon mucosa. Characterization of T-cells and cytokines production; T_H1 and T_H17 clones expansion and cytokines production, considering also T_H2 clone and T_H2-related cytokines production; evaluation of eosinophils, mast cells, macrophages, endothelial cell characteristics and cytokines/mediators production
	Toll-like receptor evaluation
Cholinergic activation, increased acetylcholine releasing from the myenteric plexus, increased smooth muscle contractility, epithelial prosecretory state, rise of luminal water content	Ultrasound evaluation of intestinal loop, before and after wheat/gluten challenge
Search for serum markers of gluten sensitivity	Performing *in vitro* flow cytometric basophil activation test with wheat components and serum-specific immunoglobulin G assays for food allergens to better explore the allergic gluten sensitivity hypothesis
Activation of hormonal responses to wheat/gluten exposure	Evaluation of hormonal response; for example, hypothalamic–pituitary–adrenal axis, renin–angiotensin system, hypothalamic–pituitary–gonadal axis.

0 according to the Marsh-Oberhüber modified classification). The remaining 40% of patients have a mild increase in IELs of up to 40% (grade 1), which is lower than the IEL percentage usually found in CD patients [11,63,65]. Nonetheless, grade 1 lesions are known to occur not only in gluten-related conditions but also in a wide array of diseases; for example, food allergies, common variable immunodeficiency, intestinal infections, *Helicobacter pylori* infection, and autoimmune disorders (such as Hashimoto thyroiditis and type 1 diabetes mellitus) [124–126]. In the context of a grade 1 lesion, EMA detection in the intestinal mucosa culture medium would suggest a diagnosis of potential CD rather than NCGS [110,111]. Finally, as recently suggested, it might be useful to determine duodenal and/or ileum–colon intraepithelial and lamina propria eosinophil counts, especially when there is suspicion of allergic NCGS patients (i.e., with multiple food allergy, including NCGS) [87]. WA patients should be excluded by skin prick testing and serum IgE antibodies specific to gluten and wheat fractions [127,128]. Figure 4 shows a diagnostic flowchart for NCGS and CD.

NATURAL HISTORY, PROGNOSIS, AND THERAPY OF NCGS

Knowledge about NCGS natural history and outcome is still lacking. Whether patients with NCGS are at risk of complications, such as intestinal lymphoma or other gastrointestinal neoplasm, is yet to be determined. Similar to CD patients, NCGS patients should change their dietary habits and consume foods with minimal gluten content. Cereals, such as buckwheat, rice, corn, and millet, and vegetables, such as quinoa, amaranth, and soybean, are recommended as substitutes for gluten-containing products. Commercially available gluten-free products used by CD patients can be proposed to NCGS patients to achieve a thoroughly gluten-free regimen. Considering the lack of knowledge as to whether NCGS is a permanent or a transient condition, periodic reintroduction of gluten (yearly?) on GFD might be advised [9,10,23–25].

CONCLUSIONS

Patients sensitive to dietary gluten are increasingly recognized in daily clinical practice. As a result of the broad symptom spectrum, NCGS might be regarded as a syndrome, rather than a gastrointestinal disease. Indeed, IBS-like and extraintestinal, mainly neurological, symptoms improve or disappear upon gluten withdrawal and recur when gluten-containing foods are reintroduced into the patient's diet. To date it has been shown that patients suffering from NCGS are a heterogeneous group, composed of several subgroups each characterized by different pathogenesis, clinical history, and probably clinical course. NCGS diagnosis should be corroborated by CD and wheat allergy exclusion, along with a personal history of food allergy in infancy, coexistent atopy, and positive IgG AGA and flow cytometric basophil activation test with wheat and duodenal and/or ileum–colon intraepithelial and lamina propria eosinophil counts. However, future research should aim to identify reliable biomarkers for NCGS diagnosis and to better define the different NCGS subgroups (Table 3).

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Received March 29, 2013; revision accepted August 13, 2013.