Cellular & Molecular Immunology (2013) 10, 383–392

www.nature.com/cmi

REVIEW

Non-celiac gluten sensitivity: questions still to be answered despite increasing awareness

Umberto Volta, Giacomo Caio, Francesco Tovoli and Roberto De Giorgio

Recently, the increasing number of patients worldwide who are sensitive to dietary gluten without evidence of celiac disease or wheat allergy has contributed to the identification of a new gluten-related syndrome defined as non-celiac gluten sensitivity. Our knowledge regarding this syndrome is still lacking, and many aspects of this syndrome remain unknown. Its pathogenesis is heterogeneous, with a recognized pivotal role for innate immunity; many other factors also contribute, including low-grade intestinal inflammation, increased intestinal barrier function and changes in the intestinal microbiota. Gluten and other wheat proteins, such as amylase trypsin inhibitors, are the primary triggers of this syndrome, but it has also been hypothesized that a diet rich in fermentable monosaccharides and polyols may elicit its functional gastrointestinal symptoms. The epidemiology of this condition is far from established; its prevalence in the general population is highly variable, ranging from 0.63% to 6%. From a clinical point of view, non-celiac gluten sensitivity is characterized by a wide array of gastrointestinal and extraintestinal symptoms that occur shortly after the ingestion of gluten and improve or disappear when gluten is withdrawn from the diet. These symptoms recur when gluten is reintroduced. Because diagnostic biomarkers have not yet been identified, a double-blind placebo-controlled gluten challenge is currently the diagnostic method with the highest accuracy. Future research is needed to generate more knowledge regarding non-celiac gluten sensitivity, a condition that has global acceptance but has only a few certainties and many unresolved issues.
Cellular & Molecular Immunology (2013) **10,** 383–392; doi:10.1038/cmi.2013.28; published online 12 August 2013

Keywords: celiac disease; epithelial barrier function; gut inflammation; non-celiac gluten sensitivity; wheat allergy

INTRODUCTION

Growing evidence indicates that a marked increase in gluten-related disorders has been observed in recent years.[1,2] Many factors have contributed to the development of gluten-related pathology, starting with the worldwide spread of the Mediterranean diet, which is based on a high intake of gluten-containing foods. In the Mediterranean area, the mean daily gluten consumption is particularly high (approximately 20 g and even higher in some countries).[3] Moreover, the mechanization of farming and the growing industrial use of pesticides have favored the development of new types of wheat with a higher amount of toxic gluten peptides that cause the development of gluten-related disorders.[4] In addition, bread and bakery products currently contain a higher quantity of gluten than in the past due to the reduced time of dough fermentation.[5] It must also be noted that diagnostic tools for gluten-induced disorders, such as celiac disease and wheat allergy, have progressively improved.[6,7]

Gluten is the main protein complex of wheat and other cereals, including barley, rye and spelt. When gluten-containing flours are kneaded with water, gliadins and glutenins, the major components of gluten, provide viscosity and elasticity to the dough.[8] These proteins are resistant to gastric digestion and increase the permeability within the small intestine through cytoskeletal rearrangement, overexpression of zonulin and tight junction dysfunction.[9,10] Small-intestine homeostasis is altered by gluten proteins through the inhibition of epithelial cell growth and the induction of apoptosis.[11]

Gluten initiates a wide array of disorders, such as celiac disease, wheat allergy and gluten-related ataxia and peripheral neuropathy.[2] Celiac disease is an autoimmune disorder with a well-characterized autoantigen (tissue transglutaminase). The current model of celiac disease is the result of significant advances in our understanding of its pathogenic mechanisms. Moreover, the availability of highly sensitive diagnostic tests and more detailed histopathological criteria has completely

Department of Medical and Surgical Sciences, St. Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy
Correspondence: Dr U Volta, Laboratory of Immunology, Department of Medical and Surgical Sciences, University of Bologna, St. Orsola-Malpighi Hospital, Via Massarenti, 9, 40138 Bologna, Italy.
E-mail: umberto.volta@aosp.bo.it
Received: 19 March 2013; Revised: 30 May 2013; Accepted: 31 May 2013

changed the clinical 'scenario' of celiac disease, allowing for the identification of groups that are at risk for celiac disease.[6]

Wheat allergy is defined as an adverse immunological reaction (IgE- and non-IgE-mediated) to gluten and other proteins contained in wheat.[7] Depending on the route of allergen exposure and the underlying immunological mechanisms, wheat allergy is classified as a classic food allergy (affecting the skin, gastrointestinal and respiratory tract), wheat-dependent exercise-induced anaphylaxis, occupational asthma (so-called baker's asthma) and rhinitis, and contact urticaria.

The spectrum of gluten-related disorders has recently acquired a new member, represented by non-celiac gluten sensitivity (NCGS).[1,2] Patients with NCGS test negative for celiac disease and wheat allergy, but after eating foods containing gluten, they experience symptoms that remit after gluten exclusion from the diet and recur following gluten reintroduction. In recent years, NCGS has been regarded as an intriguing topic by researchers. Its existence was hypothesized more than 30 years ago by a double-blind crossover trial showing that six of eight women complaining of abdominal pain, bloating and diarrhea were gluten sensitive in the absence of celiac disease.[12] After 20 years with no mention of NCGS, in 2000, Kaukinen *et al.*[13] reported that 63% of 94 adults complaining of abdominal symptoms after gluten ingestion did not satisfy the diagnostic criteria for celiac disease and wheat allergy, and because they benefited from a gluten-free diet (GFD), they were regarded as affected by NCGS. These two papers remained the only reports of the possible existence of NCGS for many years, and patients with symptoms after gluten ingestion but without evidence of antitissue tranglutaminase antibodies (tTGA), small-intestine damage and IgE to wheat were advised to continue integrating gluten into their diet because gluten was not thought to be the cause of their condition. For this reason, both gastroenterologists and allergists did not treat these patients, who remained in a diagnostic no-man's land.[14] In most cases, these patients were regarded as suffering from mental disorders and were frequently referred to psychiatrists.

Over the past 5 years, there has been a resurgence in research interest regarding NCGS, as demonstrated by the two Consensus Conferences on NCGS held in London (2011) and Munich (2012) and by several scientific contributions on this topic.[15–18] Nevertheless, NCGS is still a controversial issue. On the one hand, there is the possibility that many patients display an imaginary syndrome with a subjective sensation of improvement due to the placebo effect of gluten withdrawal.[19] Many patients are deeply influenced by the fact that GFD is the latest diet craze embraced by many celebrities. On the other hand, there is a general agreement that NCGS does exist with an effective improvement of symptoms evoked by GFD.[20] However, the detection of NCGS in these cases remains highly presumptive due to the difficulty of objectively demonstrating this diagnosis in the absence of specific markers. Recently, the American press (*USA Today* and the *Washington Post*) has reported that, according to a market survey, 15%–25% of the US population (i.e., 40–70 million people) regarded a GFD as a healthy food regimen and that approximately 17 million US citizens (i.e., 6% of the US population) were suffering from NCGS, although evidence-based data on NCGS prevalence in the general population are not yet available.[16] Global sales of gluten-free foods are expected to reach US$4.3 billion by 2015.[18] Currently, the ratio of Google to PubMed citations for NCGS is higher than 5000 : 1, thus raising the suspicion that NCGS is a problem created by the media more than an emerging clinical entity.[16]

This review discusses the current knowledge regarding NCGS, defining its basic immunological mechanisms, pathogenesis, clinical aspects and diagnostic criteria. Our aim is to provide a practical appraisal of NCGS that is useful for general practitioners and internists in the management of this emerging gluten-related condition.

BASIC IMMUNOLOGICAL MECHANISMS AND PATHOGENESIS

Abnormalities of the immune system are responsible for the development of both NCGS and celiac disease.[1,21] In healthy individuals, the immune system, through innate and adaptive immunity, plays a central role in the maintenance of tolerance to dietary antigens and other potential harmful pathogens, thus protecting the organism from the development of diseases.[22] The innate immune response is immediate and fast, including both cellular and humoral components.[23] Several cells are involved, including macrophages, neutrophils, dendritic cells, monocytes, mast cells and natural killer T cells, expressing both natural killer receptors and CD1d-restricted αβ-T-cell receptors.[24] Another feature of innate immunity is the humoral secretion of complement proteins, C-reactive protein and lipopolysaccharide-binding protein. The adaptive immune response is characterized by a delayed onset and by memory capacity, involving both T and B cells.[25] T cells are activated after the interaction between major histocompatibility complex-bound peptides and T-cell receptors. $CD8^+$ T cells are stimulated by intracellular antigens presented by major histocompatibility complex class I molecules, which are expressed in all nucleated cells. The activation of $CD8^+$ cells induces apoptosis, which plays an essential role in defending the organism against viral infections. $CD4^+$ T helper cells are activated by interactions with major histocompatibility complex class II molecules on antigen-presenting cells and secrete cytokines that significantly influence the immune response. Based on the type of cytokine secreted, the Th cells are classified as Th1, Th2, Th17, regulatory T (Treg) and T follicular helper cells.[26,27] Th1 cells secrete interferon-γ and interleukin (IL)-2, enhancing cell-mediated responses. Moreover, Th1 cells stimulate macrophages and $CD8^+$ T cells. Th2 cells regulate humoral immunity and antibody production through the secretion of IL-4, IL-5, IL-10 and IL-13 and tumor-necrosis factor-α. Moreover, Th2 cells are involved in IgE-mediated allergy and in protecting against helminth infections. Th17 cells have a role both in host defense against pathogens and in autoimmune diseases.[28] Treg cells express the transcription factor FoxP3, limit the immune response and are important in the

regulation of immunological tolerance.[29] Moreover, Tregs secrete the cytokines TGF-β and IL-10. Follicular helper T-cells express IL-21 and differentiate B cells into memory B cells and plasma cells.[27]

The loss of immune homeostasis accompanied by the activation of innate/adaptive immunity is the first step for the appearance of immune disorders. Both celiac disease and non-celiac gluten sensitivity share an enhanced innate immune response. Gluten and its related peptides are the triggers, breaking immunological tolerance by inducing the innate immune response and stimulating dendritic cells, which results in leukocyte infiltration and inflammation of gut mucosa. The immune mechanisms underlying celiac disease also include the activation of adaptive immunity by tissue transglutaminase TG2, the celiac autoantigen, and through the deamidation of gluten peptides. Gluten-reactive T cells are present in the lamina propria of patients with celiac disease and preferentially recognize deamidated gluten peptides in the context of disease-associated histocompatibility leukocyte antigen (HLA) molecules.[21] Several studies have confirmed the central role of adaptive immunity in the development of celiac disease by showing systemic and mucosal expression of cytokines associated with Th1 and Th17 responses.[30–33] In contrast to celiac disease, an overexpression of adaptive immunity markers has not been found in NCGS. In particular, IL17A, IL-6, interferon-γ, IL-17 and IL-21 were not increased in intestinal biopsies of NCGS patients.[34,35]

These findings are in line with the hypothesis that NCGS is mostly supported by innate immune mechanisms. The behavior of Toll-like receptors (TLRs), a class of proteins that play a key role in the innate immune system as well as the digestive system, supports this view. TLRs are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells, such as macrophages and dendritic cells, which recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers, such as the skin or intestinal mucosa, they are recognized by TLRs, which activate immune cell responses. Small-intestine expression of TLR2 and, to a lesser extent, of TLR1 and TLR4 is greater in patients with NCGS than in celiac patients.[34] NCGS also differs from celiac disease in the small-intestine expression of the T_{REG} marker, FOXP3, which is markedly weaker in gluten-sensitive patients than in celiac patients.[34] Although the reduction of mucosal FOXP3 expression has been regarded as a sign of the loss of immune homeostasis, which favors the development of autoimmune conditions, in other studies this immune marker was found to be markedly up-regulated in the blood and intestinal mucosa of celiac patients.[36,37] Therefore, the reduced expression of this Treg marker in NCGS could be interpreted in the context of a reduced activation of adaptive immunity relative to celiac disease.

In contrast to celiac disease, for which the main pathogenic mechanisms have been extensively elucidated over the years, the pathogenesis of NCGS is still poorly defined (Figure 1). A

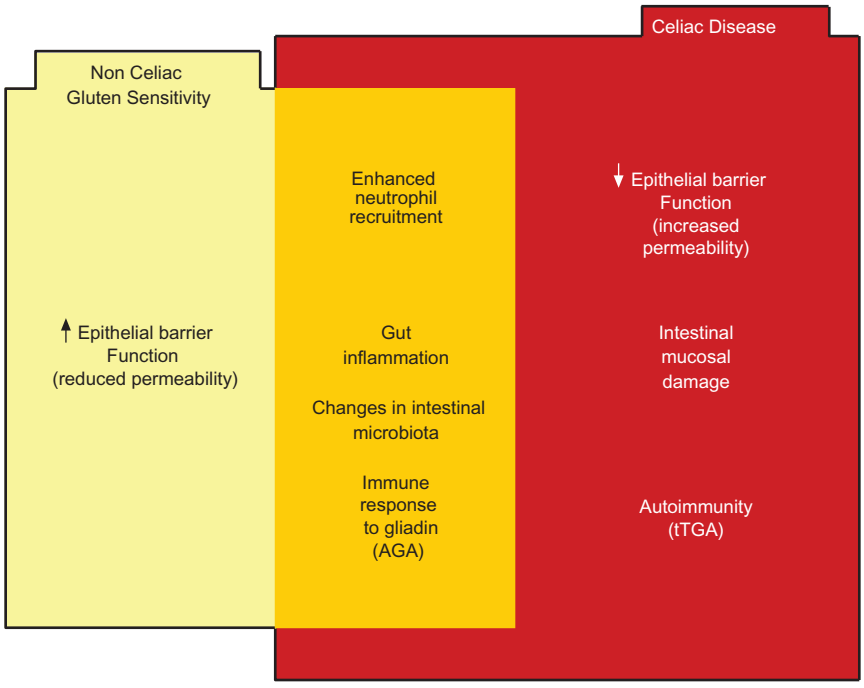

Figure 1 Pathogenic mechanisms of NCGS. Enhanced neutrophil recruitment, gut inflammation, changes in intestinal microbiota and immune response to gliadin are features (orange field) common to NCGS and celiac disease. Decreased intestinal barrier function (increased permeability), intestinal mucosa damage and autoimmunity expressed by immune reaction to tissue transglutaminase are typical of celiac disease (red field), whereas enhanced intestinal barrier function (reduced permeability) has been demonstrated in NCGS. AGA, anti-gliadin antibodies; NCGS, non-celiac gluten sensitivity; tTGA, tissue transglutaminase antibodies.

pathophysiological aspect that has been investigated by researchers is the epithelial barrier function of the intestinal mucosa.[1] It has been hypothesized that changes in the intestinal barrier function might help to differentiate NCGS from celiac disease. The loss of the intestinal barrier function with a marked increase in permeability has been clearly demonstrated in celiac disease.[38] Different form celiac patients, NCGS subjects display a reduced intestinal permeability, as measured by the lactulose/mannitol absorption test, suggesting an increased barrier function in these patients.[34] Moreover, expression of claudin-4 mRNA is significantly greater in duodenal biopsies from NCGS patients than in those from celiac patients.[34] Because increased claudin-4 levels are an indicator of reduced intestinal permeability, this observation is in line with the hypothetical reduced permeability of the intestinal barrier in NCGS. This hypothesis has been questioned by Biesekierski *et al.*,[39] who did not find any significant difference in the intestinal barrier function of two randomly treated groups of NCGS patients (one challenged by gluten, the other by placebo) using the dual sugar absorption test. Different results from those reported by Sapone *et al.* have also been obtained by Vazquez-Roque *et al.*,[40] who have documented increased intestinal permeability in a subgroup of HLA-DQ2/DQ8$^+$ NCGS patients with irritable bowel syndrome, diarrhea and gluten sensitivity. Further studies are needed to establish whether the epithelial barrier function in NCGS patients is different from that of celiac disease patients.

New insights to explain the pathogenic mechanisms of NCGS have been provided by HLA-DQ8 transgenic mice sensitized to gliadin. Using this experimental model, an increased secretion of acetylcholine from the myenteric plexus resulting in enhanced muscle contractility and epithelial hypersecretion has been demonstrated. Gluten withdrawal reverted both abnormalities.[41] Moreover, differences in luminal antigens caused by an increased amount of Gram-negative bacteria and a decreased number of Gram-positive Lactobacilli in the intestinal microbiota triggered the inflammatory response to dietary antigens, such as gluten.[42] Based on these findings, it is plausible to hypothesize that neuromuscular dysfunction and gut microbiota could have a role in gluten-induced symptoms.

Recent studies have shown that gluten and its related proteins are not the only triggers of NCGS and that other wheat proteins likely play a relevant role in causing this syndrome (Figure 2). In particular, the attention of researchers has been focused on amylase–trypsin inhibitors, which are strong activators of the innate immune responses of monocytes, macrophages and dendritic cells.[43] Amylase–trypsin inhibitors could fuel inflammation and immune reactions in several intestinal and non-intestinal immune disorders, including NCGS, celiac disease and Baker's asthma.

Many patients with NCGS display multiple food hypersensitivities, which could in part be related to a diet rich in fermentable oligo-, di- and mono-saccharides and polyols (FODMAPs).[44] FODMAPs are poorly absorbed short-chain carbohydrates that cause distension of the intestinal lumen

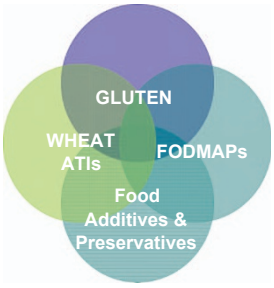

Figure 2 Known triggers of NCGS. Gluten is the primary trigger but not the only trigger of this syndrome because other wheat proteins, such as the wheat ATIs, have been demonstrated to elicit the innate immune response leading to NCGS. Moreover, many NCGS pts display a food hypersensitivity elicited by FODMAPs. Functional gastrointestinal symptoms observed in patients with NCGS as well as with other disorders, including IBS, could also be partly related to food additives, such as glutamates, benzoates, sulfites and nitrates, which are added to many commercial products for a variety of reasons (e.g., to enahnce flavor, color and preservative function). FODMAP, fermentable oligo-, di- and mono-saccharide and polyol; IBS, irritable bowel syndrome; NCGS, non-celiac gluten sensitivity; ATIs, amylase trypsin inhibitors.

with liquid and gas due to their small molecular size and rapid fermentability and lead to functional gastrointestinal symptoms. Common food sources of FODMAPs are grains and cereals (wheat, rye and barley), milk, legumes, honey, fruits (watermelon, cherry, mango and pear) and vegetables (chicory, fennel, beetroot and leek). A low FODMAP diet significantly improves the functional gastrointestinal symptoms in NCGS patients. Functional gastrointestinal symptoms observed in patients with NCGS as well as other disorders, including irritable bowel syndrome (IBS), could also be partly related to food additives, such as glutamates, benzoates, sulfites and nitrates, which are added to many commercial products for different reasons (to improve flavor and color and to preservative function). In general, the stronger the flavor of the food, the higher the chemical content will be. Food chemicals add strong afferent stimuli to the enteric nervous system. When patients display visceral hypersensitivity, normal physiological stimulation by such chemicals may result in exaggerated effector responses leading to luminal distension. Therefore, a low-FODMAP diet with a low content of additives and preservatives is a realistic and efficacious attempt for improving gastrointestinal symptoms in NCGS patients.

Another factor that could play a role in NCGS development could be the opioid-like activity of gluten. Indeed, gluten proteins can mimic some of the effects of opiates by altering the intestinal transit time in healthy volunteers in a naloxone-reversible manner.[45] Finally, it must be emphasized that in many circumstances NCGS is an imaginary ailment that is caused by the nocebo effect of gluten ingestion. This possibility in patients with a self-diagnosis of food hypersensitivity has clearly been established by double-blind trials.[46] The placebo effect of the elimination diet is generally regarded as superior to that of drug treatment.

THE EPIDEMIOLOGICAL AND CLINICAL PICTURE

The prevalence of NCGS has yet to be defined because no reliable epidemiological study has been published to date. Significantly different prevalences have been reported in primary and tertiary care. The National Health and Nutrition Examination Survey, a program involving primary care surveillance that was designed to assess the health and nutritional status of adults and children in the United States and was performed by means of interviews and physical examinations, has identified 49 cases of suspected NCGS over 7762 patients that were examined (age range: 6–80 years) over the period of 2009–2010, with a prevalence of 0.63%. In tertiary care at the Center for Celiac Research, University of Maryland, the criteria for NCGS were recognized in 347 of 5896 patients observed between 2004 and 2010, with a prevalence of 6%.[2] Therefore, by the extrapolation of these results, a highly variable proportion of the US population ranging from 0.63% (approximately 2 million) to 6% (approximately 16 million) should be affected by NCGS. Although it is expected that the majority of patients with gluten-related symptoms are referred to specialist centers, this referral bias alone cannot explain the broad difference reported in the two above-mentioned studies. In a recent survey performed in the general population of the United Kingdom by means of a medical questionnaire, 139 of 1002 people (13%) complained of gluten-related symptoms. Approximately 80% of people reporting gluten-induced symptoms were female. Patients with irritable bowel syndrome, a common intestinal disorder with a prevalence of 15%–20% in the general population, more frequently reported being gluten sensitive than those without IBS (35% *versus* 11%, $P<0.0001$). The close linkage between NCGS and IBS has been underlined by Carroccio *et al.*,[47] who found that 30% of 920 patients with IBS had NCGS associated with multiple food hypersensitivity in the majority of cases. Our personal experience with NCGS prevalence based on the ratio of the new cases of suspected NCGS to the new cases of celiac disease observed over a period of 12 months suggests that NCGS is slightly more frequent than celiac disease (ratio: 1.6 : 1) (unpublished data).

From a clinical point of view, NCGS is regarded as a syndrome that is characterized by a wide array of gastrointestinal and extra-intestinal symptoms that occur shortly after the ingestion of gluten, improve or disappear when gluten is withdrawn from the diet and recur when gluten is reintroduced. Ruling out celiac disease by means of negative serology and the absence of celiac histological findings as well as ruling out wheat allergy by means of negative testing for specific IgE and/or prick tests to wheat are prerequisites for the diagnosis of NCGS.[1,2]

The time interval between gluten ingestion and the appearance of symptoms in NCGS varies from a few hours to a few days, which is quite different from that observed with both wheat allergy and celiac disease. Indeed, in wheat allergy, symptoms usually appear in a few minutes after gluten exposure, whereas in celiac disease, the time interval between gluten ingestion and the clinical manifestation can be long (up to weeks or years).[1,2]

One of the few clinical studies on NCGS published to date comes from our group.[48] We studied 78 patients (56 females and 22 males; median age: 38 years, range: 17–63 years) with NCGS diagnosed in the Celiac Disease Unit of Bologna University between January 2009 and June 2011. In all 78 patients, NCGS was suspected on the basis of symptoms with an early onset after gluten ingestion. Celiac disease and wheat allergy were excluded by means of serology (anti-endomysial–EmA- and tissue transglutaminase antibodies–tTGA-), duodenal biopsy and specific IgE/Prick tests to wheat in all patients. NCGS diagnosis was confirmed by a trial of GFD for 6 months with a quick disappearance of symptoms, followed by an open gluten challenge of 1 month with an immediate relapse of the clinical picture. All NCGS patients showed both gastrointestinal and extra-intestinal symptoms occurring within a few hours or days of gluten ingestion. The most frequent gastrointestinal symptoms were abdominal pain and bloating, often associated and present in 77% and 72% of cases, respectively, followed by diarrhea (40%) and constipation (18%). Approximately 20% of our patients complained of gastroesophageal reflux disease and 10% of our patients complained of aphthous stomatitis. Among extra-intestinal signs, the most frequent symptom was mental confusion or a 'foggy mind', defined as a sensation of lethargy elicited by gluten, observed in 42% of cases, followed by fatigue (36%), skin rash (33%), headache (32%), joint and muscle pain (fibromyalgia-like syndrome) (28%), leg or arm numbness (17%), depression and anxiety (15%) and anemia (15%). The same symptoms with similar frequencies have been reported in NCGS patients diagnosed in the Maryland Center[2] (Table 1). The clinical features of NCGS as determined from the already published studies show that this syndrome is rare in infancy and is more frequent in adolescents and adults, with a high number of cases diagnosed in the elderly. Moreover, similar to celiac disease, NCGS is much more frequent in females than in males. The preliminary results of a prospective survey promoted by the Italian Association for Celiac Disease in Italian Centers for the diagnosis of gluten-related disorders has identified 200 new cases of NCGS over a few months, showing that the median age of disease onset was 55 years (range: 18–80 years), with a markedly higher prevalence in females than in males (F/M 6 : 1).[49] In the majority of patients with NCGS, the previously absent gluten-related symptoms usually appear some months or years before the diagnosis is suspected. In approximately 50% of NCGS patients, the condition coexists with irritable bowel syndrome and other food intolerances, such as lactose and fructose intolerance, in a significant number of cases. Another relevant clinical aspect of NCGS is its frequent occurrence in first degree relatives of celiac disease patients who often display the evidence of immune responsiveness to gluten despite having normal

Table 1 Clinical presentation of non-celiac gluten sensitivity (NCGS)

	Center for Celiac Research, University of Maryland, US 347 NCGS pts diagnosed between 2004 and 2010[2]	*Center for Celiac Research, University of Bologna, Italy 78 NCGS pts diagnosed between 2009 and 2011*[48]
Bloating	72%	72%
Abdominal pain	68%	77%
Diarrhea	33%	40%
Constipation	n.r.	18%
Eczema and/or rash	40%	33%
Headache	35%	32%
Foggy mind	34%	42%
Fatigue	33%	36%
Depression/ anxiety	22%	15%
Anemia	20%	15%
Numbness in legs, arms and fingers	20%	17%
Joint/muscle pain	11%	28%

Abbreviations: NCGS, non-celiac gluten sensitivity; n.r.: not reported.

small-intestine mucosa. This observation has been confirmed by the demonstration that in our previous study 10 of 78 (12.8%) NCGS patients were relatives of celiac patients.[48] A rectal gluten challenge proved to be a valid tool for detecting the mucosal findings suggestive of gluten sensitivity in the relatives of celiac patients.[50]

Whether NCGS patients are at risk for associated autoimmune disorders and complications such as celiac disease has not been established. Preliminary data suggest that the presence of autoimmune disorders in NCGS would be a rare event. Indeed, in our group of 78 NCGS patients, none had type 1 diabetes mellitus and only one (1.3%) had autoimmune thyroiditis, compared with 5% and 19%, respectively, of 80 patients with celiac disease.[48] Another hot topic is whether patients with NCGS are at risk of complications that are similar to those that occur with celiac disease, such as ulcerative jejuno-ileitis, collagenous sprue, small-intestine lymphoma and other gastrointestinal neoplasms. The follow-up period with NCGS patients is still too short for any conclusion to be drawn regarding the outcome of this condition.

DIAGNOSTIC CRITERIA

Because of the lack of a specific biomarker, the diagnosis of NCGS relies on the accurate assessment of clinical features along with the exclusion of wheat allergy and celiac disease (Table 2). The exclusion of gluten from the diet is followed by significant improvements, including the disappearance of intestinal and extra-intestinal symptoms, and gluten reintroduction causes symptom recurrence. Symptom improvement or cessation, as well as their reoccurrence attributable to the absence or presence of dietary gluten, is suggestive of NCGS. However, as a placebo effect induced by gluten withdrawal cannot be excluded, double-blind, placebo-controlled challenge trials are strongly recommended to confirm the NCGS diagnosis. Two of these trials have already been performed, confirming the existence of NCGS. Biesiekierski *et al.*[39] showed that the double-blind challenge caused the recurrence of clinical symptoms in 68% of patients receiving gluten versus 40% of those receiving the placebo. Similar results were obtained by Carroccio *et al.*,[47] whose double-blind placebo-controlled challenge confirmed a significant worsening of intestinal and extra-intestinal symptoms in the gluten *vs.* the placebo group.

As noted above, before considering NCGS, a physician should exclude both wheat allergy and celiac disease, using appropriate tests performed under a gluten-containing diet. Wheat allergy should be ruled out by testing for serum IgE antibodies to gluten and wheat fractions as well as skin-prick tests, whereas celiac disease must be excluded by the absence of specific serological tests, such as IgA tTGA, IgA EmA and IgG deamidated gliadin peptide antibodies.[6,7] The only serological marker found in patients with NCGS is the first-generation antibody to gliadin (AGA).[47,48,51] AGA positivity of the sera of about half of the NCGS patients has been found, and these antibodies are almost always confined to the IgG class, only occasionally belonging to the IgA class.[48] In the 78 NCGS patients studied in our center, AGA IgG were detected in

Table 2 Diagnostic criteria for non-celiac gluten sensitivity

- Gluten ingestion typically elicits the rapid occurrence (in a few hours or days) of intestinal and extra-intestinal symptoms (Table 1)
- Symptoms disappear quickly (in a few hours or days) after the elimination of gluten from the diet
- Reintroduction of gluten causes the rapid recurrence of symptoms
- Celiac disease must be ruled out by means of negative serology (endomysial and tissue transglutaminase IgA antibodies) and a duodenal biopsy on a gluten-containing diet
- Wheat allergy tests (specific IgE as well as skin prick tests), performed on a gluten-containing diet, must be negative
- A double-blind, placebo-controlled gluten challenge test is needed in each suspected patient to confirm the diagnosis and to exclude a placebo effect induced by gluten exclusion

(i) Although no serological marker is available for non-celiac gluten sensitivity (NCGS), it must be emphasized that approximately 50% of NCGS pts are positive for first-generation anti-gliadin antibodies (AGA), mainly IgG; (ii) NCGS is unrelated to the celiac disease genetic markers (i.e., HLA-DQ2 and -DQ8), which are found in approximately 40% of NCGS patients *vs.* 30% in the general population.

56% of NCGS patients in comparison with their positivity in 81% of celiac cases, and antibody titers in NCGS patients were as high as those found in celiac disease. AGA IgA had a very low prevalence in NCGS patients (8%), with very low titers in comparison with those found in celiac disease.[48] Although AGA is not a specific test for NCGS because these antibodus are present in many other conditions, such as autoimmune liver diseases, irritable bowel syndrome, connective tissue disorders and even blood donors, for the time being, the positivity of these antibodies (especially at a high titers) in patients with suspected NCGS can contribute to this diagnosis.[52] AGA IgG disappeared in 19 of 20 patients with NCGS within 6 months of initiating a GFD, whereas they remained positive in about half of CD patients after gluten withdrawal.[1,53] It is reasonable to hypothesize that immunological memory might be active in celiac disease but not in NCGS.

A duodenal biopsy is highly recommended in patients with suspected NCGS when they are on a gluten-containing diet to definitively rule out a celiac disease diagnosis, even if the celiac serology is negative, because of the possibility of seronegative celiac disease, which occurs in 1%–2% of the total cases of celiac disease.[6] There is a wide consensus that NCGS patients have low-grade inflammation in their small intestines, but the majority (approximately 60%) display a normal histology of duodenal mucosa with a number of intraepithelial lymphocytes (IELs) lower than 25 per 100 epithelial cells (grade 0 according to Marsh–Oberhuber modified classification).[2,48] The remaining 40% have a mild increase in IELs of up to 40% epithelial cells (lesion grade 1), which is less than the percentage of IELs that are usually detected in celiac patients.[54] In contrast to celiac disease, in NCGS, there is not an increase of T-cell receptor γ/δ IELs.[34]

NCGS does not correlate with the HLA-DQ2 and -DQ8, which are markers of celiac disease. Positivity for HLA-DQ2 and/or -DQ8 was found in 46% of NCGS patients. This figure is much lower than that found in patients with CD (99%) and is comparable to that of the general population (30%).[48]

TREATMENT

The diagnosis of NCGS should be confirmed by a double-blind, placebo-controlled gluten challenge.[39,47] After confirmation of the NCGS diagnosis by this procedure, patients must change their dietary habits and consume foods with a minimal gluten content. Cereals such as rice, corn, buckwheat and millet and leguminosae such as quinoa, amaranth and soybean are recommended as substitutes for gluten-containing products. Commercially available gluten-free products are useful for patients with NCGS to achieve a thoroughly gluten-free regimen, but, as also recommended for celiac disease patients, naturally gluten-free foods, such as meat, fish, eggs, fruit and vegetables, should be integrated into their diets to ensure proper nutrition. In the entire diet regimen, the use of commercially available gluten-free products should remain low to limit the introduction of chemical additives and preservatives, which are abundant in these products and a potential cause of functional gastrointestinal symptoms. In contrast to patients with celiac disease, NCGS patients should not fear gluten contamination due to traces of gluten inadvertently introduced by foods. However, it must be mentioned that the level of tolerance varies across individuals and there are also patients with NCGS who do not tolerate very small amounts of gluten. GFD leads to the complete disappearance of symptoms in most patients with NCGS, whereas in other cases the improvement after gluten withdrawal is only partial. Because no clues exist as to whether gluten sensitivity is a permanent or transient condition, the reintroduction of gluten after 1–2 years on GFD could be advised. A correct approach may be a desensitization trial with the introduction of progressively small amounts of gluten.

CONCLUSIONS

The recent increase in the number of patients sensitive to dietary gluten without evidence of celiac disease and wheat allergy has contributed to the recognition of a new gluten-related syndrome classified as NCGS.[1,2] Although the existence of NCGS has been accepted by the scientific community, our knowledge about NCGS is still limited, and there are many unsettled points that must be clarified. The hypothesized involvement of several pathogenic mechanisms and the large variability of the clinical picture have raised the idea that NCGS is an umbrella covering different subtypes of illnesses rather than a single entity. Innate immunity plays a major role as a trigger of this syndrome, but several other relevant factors are likely involved in its pathogenesis, including low-grade inflammation in the intestinal mucosa, changes in intestinal permeability and alterations of the intestinal microbiome.[16,34,35,42] Although a conceptual link between gluten and the generation of symptoms has been clearly proved in NCGS patients, other proteins contained in wheat and bread have the potential to cause the symptoms that are experienced by patients who have this syndrome. In this context, wheat amylase–trypsin inhibitors, a complex of proteins highly resistant to intestinal proteases and that elicit innate immunity, could represent a trigger for gluten sensitivity.[43] Gluten is the sole cause of symptoms in only a small subgroup of NCGS patients; in the majority of these patients, multiple food hypersensitivity underlies the clinical picture.[47] In this context, a diet rich in FODMAPs, present not only in gluten-containing cereals but also in milk, honey and legumes might elicit this syndrome.[44] Moreover, chemical additives, such as glutamates, benzoates, sulfites and nitrates, which are added to many commercial products for various reasons (to improve flavor, color and preservative function), might have a role in evoking the functional gastrointestinal symptoms of NCGS and other disorders characterized by intestinal inflammation, such as irritable bowel syndrome.[44] In this respect, a subgroup of NCGS patients does not improve by eating commercially gluten-free products that are rich in additives and preservatives, and they experience the resolution of gluten-related symptoms by following a diet based on naturally gluten-free foods (Volta *et al.*, unpublished data).

Table 3 Comparison between celiac disease and non-celiac gluten sensitivity features

	Celiac disease	*Non-celiac gluten sensitivity*
Epidemiology	1%	To be defined (range 0.63%–6%)
Duration	Permanent	Unknown
Prevalent immune pathogenic mechanism	Adaptive immunity	Innate immunity
Onset	At any age	Adults (rare in pediatric age)
Sex	Female/male ratio 2 : 1	Female/male ratio >3 : 1
Time interval between gluten ingestion and symptoms	Weeks to years	Hours or a few days
Clinical picture	Intestinal and extraintestinal (systemic)	Intestinal and extra-intestinal (mainly neurological)
Biomarkers	tTGA, EmA, DGP	None (positivity for AGA in approximately 50% of cases but low specificity)
Genetics	HLA-DQ2 and -DQ8 linked	No known genetic link
Duodenal histology	From mild lesions to villous atrophy	Normal or less frequently mild lesions
Familiarity	3%–17% of first degree relatives are celiacs	Unknown, but more than 10% of NCGS pts have a relative with celiac disease
Autoimmune disorders	Frequent association (present in 10%–25% of celiac patients)	Unknown (a longer follow-up is needed)
Outcome (complications)	Refractory celiac disease, lymphoma, small-bowel carcinoma (rare (<1%) but with a poor prognosis)	Unknown (a longer follow-up is needed)

AGA, anti-gliadin antibodies; DGP, deamidated gliadin peptide antibodies; HLA, histocompatibility leukocyte antigen; NCGS, non-celiac gluten sensitivity; tTGA, tissue transglutaminase.

The heterogeneity of NCGS patients is confirmed by the various combinations of the genetic, histological and serological features of these patients. A proportion of NCGS patients display positivity for HLA-DQ2 and/or -DQ8 and/or positivity for AGA and/or mild intestinal damage (Marsh 1 lesion), which, taken together, are non-diagnostic for celiac disease but could theoretically represent the first step toward diagnosing future celiac disease. Although NCGS and celiac disease are likely two separate entities with different primary pathogenic pathways represented by innate immunity and adaptive immunity, respectively, the possibility that NCGS may evolve into celiac disease cannot be dismissed. The detection of IgA deposits by small-intestine biopsies might help to identify NCGS patients who are at risk of developing celiac disease.[55]

Another relevant point that must be clarified is whether NCGS can be associated with the development of autoimmune disorders and can develop complications (ulcerative jejunoileitis intestinal lymphoma and small-bowel carcinoma) similar to celiac disease. The clinical study of NCGS is still too limited to answer this question, which can be elucidated only through the prolonged follow-up of NCGS patients.

Although it remains unclear whether NCGS is a transient or a permanent condition, it is tempting to attempt desensitization by reintroducing small amounts of gluten in these patients. Table 3 summarizes the main differences between celiac disease and NCGS.

In conclusion, there is general agreement in the scientific community that additional studies are needed to shed light on NCGS, a condition that has been globally accepted but possesses few certainties and many questions (Table 4). Current NCGS diagnosis facilities are comparable to those for celiac disease in the early 1970s, when no marker was available for identifying celiac disease objectively. In the near future, advances that will help to provide a specific biomarker for NCGS diagnosis are expected, which happened for celiac disease by means of serology (EmA and tTGA). Currently, in the absence of a specific diagnostic test, the only correct approach for confirming the diagnosis of suspected NCGS is a double-blind placebo-controlled gluten

Table 4 Future research in NCGS

- Definition of biomarkers for NCGS (identification of specific antibodies, cytokines, chemokines)
- Evaluation of intestinal permeability by means of highly sensitive tests to definitively establish whether intestinal barrier function in NCGS is increased (as hypothesized by studies performed up to now) or decreased (as already demonstrated in celiac disease)
- Characterization of the low-grade inflammation and mild histological lesions found in NCGS patients (differences, if any, between IELs disposition in NCGS *vs.* celiac disease)
- GWAS to define whether NCGS displays some genetic link

GWAS, genome-wide association study; IEL, intraepithelial lymphocyte; NCGS, non-celiac gluten sensitivity.

challenge test, which is the only way to exclude a placebo effect induced by gluten exclusion.

1 Volta U, de Giorgio R. New understanding of gluten sensitivity. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 295–299.
2 Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PH, Hadijvassiliou M *et al*. Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Med* 2012; **10**: 13.
3 Gibert A, Espadaler M, Angel Canela M, Sánchez A, Vaqué C, Rafecas M. Consumption of gluten-free products: should be the threshold value for traces amounts of gluten be at 20, 100 or 200 p.p.m.? *Eur J Gastroenterol Hepatol* 2006; **18**: 1187–1195.
4 Belderok B. Developments in bread-making processes. *Plant Food Hum Nutr* 2000; **55**: 1–86.
5 Gobbetti M, Rizzello CG, Di Cagno R, de Angelis M. Sordough lactobacilli and coeliac disease. *Food Microbiol* 2007; **24**: 187–196.
6 Volta U, Villanacci V. Celiac disease: diagnostic criteria in progress. *Cell Mol Immunol* 2011; **8**: 96–102.
7 Inonata N. Wheat allergy. *Cur Opin Aller Clin Immunol* 2009; **9**: 238–243.
8 Blomfeldt TO, Kuktaite R, Johansson E, Hedenqvist MS. Mechanical properties and network structure of wheat gluten forms. *Biomacromolecules* 2011; **12**: 1707–1715.
9 Fraser JS, Engel W, Ellis HJ, Moodie SJ, Pollock EL, Wieser H *et al*. Coeliac disease: in vivo toxicity of the putative immunodominant epitope. *Gut* 2003; **52**: 1698–1702.
10 Drago S, El Asmar R, Di Pierro M, Clemente MG, Tripathi A, Sapone A *et al*. Gliadin, zonulin, and gut permeability: effects on celiac and non-celiac mucosa and intestinal cell lines. *Scand J Gastroenterol* 2006; **41**: 408–419.
11 Dolfini E, Roncoroni L, Elli L, Fumagalli C, Colombo R, Ramponi S *et al*. Cytoskeleton reorganization and ultrastructural damage induced by gliadin in a three-dimensional *in vitro* model. *World J Gastroenterol* 2005; **11**: 7597–7601.
12 Cooper BT, Holmes GK, Ferguson R, Thompson RA, Allan RN, Cooke WT. Gluten-sensitive diarrhea without evidence of celiac disease. *Gastroenterology* 1980; **79**: 801–806.
13 Kaukinen K, Turjanmaa K, Mäki M, Partanen J, Venäläinen R, Reunala T *et al*. Intolerance to cerealsis not specific for celiac disease. *Scand J Gastroenterol* 2000; **35**: 942–946.
14 Verdu EF, Armstrong D, Murray JA. Between celiac disease and irritable bowel syndrome: the 'no man's land' of gluten sensitivity. *Am J Gastroenterol* 2009; **104**: 1587–1594.
15 Troncone R, Jabri B. Coeliac disease and gluten sensitivity. *J Intern Med* 2011; **269**: 582–590.
16 Di Sabatino A, Corazza GR. Non-celiac gluten sensitivity: sense or sensibility? *Ann Intern Med* 2012; **156**: 309–311.
17 Lundin KEA, Alaedini A. Non-celiac gluten sensitivity. *Gastrointest Endoscopy Clin N Am* 2012; **22**: 723–734.
18 Brown AC. Gluten sensitivity: problems of an emerging condition separate from celiac disease. *Expert Rev Gastroenterol Hepatol* 2012; **6**: 41–53.
19 Godlee F. Gluten sensitivity: real or not? *BMJ* 2012; **345**: e8450.
20 Anonymus patient, Rostami K, Hogg-Kollars S. Non-coeliac gluten sensitivity: a patient's journey. *BMJ* 2012; **345**: e7982.
21 Sollid LM, Jabri B. Triggers and drivers of autoimmunity: lessons from celiac disease. *Nat Rev Immunol* 2013; **13**: 294–302.
22 Marietta EV, Murray JA. Animal models in gluten sensitivity. *Semin Immunopathol* 2012; **34**: 497–511.
23 Turvet SE, Broide DH. Innate immunity. *J Allergy Clin Immunol* 2010; **125**(Suppl 2): S24–S32.
24 Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* 2007; **25**: 297–336.
25 Bonilla FA, Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol* 2010; **125**(Suppl 2): S33–S40.
26 Zhu J, Yamane H, Paul WE. Differentiation of effector CD4T cell populations. *Annu Rev Immunol* 2010; **28**: 445–489.
27 Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011; **29**: 621–623.
28 Crome SQ, Wang AY, Levings MK. Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. *Clin Exp Immunol* 2010; **159**: 109–119.
29 Bilate AM, Lafaille JJ. Induced $CD4^+$ $FoxP3^+$ regulatory T cells in immune tolerance. *Annu Rev Immunol* 2012: **30**: 733–758.
30 Castellanos-Rubio A, Santin I, Irastorza I, Castaño L, Carlos Vitoria J, Ramon Bilbao J. TH17 (and TH1) signatures of intestinal biopsies of CD patients in response to gliadin. *Autoimmunity* 2009; **42**: 69–73.
31 Harris KM, Fasano A, Mann DL. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. *J Immunol* 2008; **181**: 4457–4460.
32 Monteleone I, Pallone F, Monteleone G. Interleukin-23 and Th17 cells in the control of gut inflammation. *Mediators Inflamm* 2009; **2009**: 297645.
33 Monteleone I, Sarra M, del Vecchio BG, Paoluzi OA, Franze E, Fina D *et al*. Characterization of IL-17A-producing cells in celiac disease mucosa. *J Immunol* 2010; **184**: 2211–2218.
34 Sapone A, Lammers K, Casolaro V, Cammarota M, Giuliano MT, de Rosa M *et al*. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. *BMC Med* 2011; **9**: 23.
35 Sapone A, Lammers KM, Mazzarella G, Mikhailenko I, Carteni M, Casolaro V *et al*. Differential mucosal IL-17 expression in two gliadin-induced disorders: gluten sensitivity and the autoimmune enteropathy celiac disease. *Int Arch AllergyImmunol* 2010; **152**: 75–80.
36 Vorobjova T, Uibo O, Heilman K, Rägo T, Honkanen J, Vaarala O *et al*. Increased FOXP3 expression in small-bowel mucosa of children with coeliac disease and type I diabetes mellitus.*Scand J Gastroenterol* 2009; **44**: 422–430.
37 Hansson T, Ulfgren AK, Lindroos E, DannAEus A, Dahlbom I, Klareskog L. Transforming growth factor-beta (TGF-beta) and tissue transglutaminase expression in the small intestine in children with coeliac disease. *Scand J Immunol* 2002; **56**: 530–537.
38 Schuppan D. Current concepts of celiac disease pathogenesis. *Gastroenterology* 2000; **119**: 234–242.
39 Biesiekierski JR, Newnham ED, Irving PM, Barrett JS, Haines M, Doecke JD *et al*. Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. *Am J Gastroenterol* 2011; **106**: 508–514.
40 Vaquez-Roque MI, Camilleri M, Smyrk T, Murray JA, Marietta E, O'Neill J *et al*. A controlled trial of gluten-free diet in patients with irritable bowel syndrome-diarrhea: effects on bowel frequency and intestinal function. *Gastroenterology* 2013; **144**: 903–911.
41 Verdu EF, Huang X, Natividad J, Lu J, Blennerhassett PA, David CS *et al*. Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G217–G225.
42 Natividad JM, Huang X, Slack E, Jury J, Sanz Y, David C *et al*. Host responses to intestinal microbial antigensin gluten sensitive mice. *PLos ONE* 2009; **4**: e6472.
43 Junker Y, Zeissig S, Kim SJ, Barisani D, Wieser H, Leffler DA *et al*. Wheat amylase trypsin inhibitors drive intestinal inflammation *via* activation of toll-like receptor 4. *J Exp Med* 2012; **209**: 2395–2408.
44 Gibson PR, Sheperd SJ. Food choice as a key management strategy for functional gastrointestinal symptoms. *Am J Gastroenterol* 2012; **107**: 657–666.
45 Corazza GR, Frazzoni M, Strocchi A, Prati C, Sarchielli P, Capelli M *et al*. Alimentary exorphin actions on motility and hormonal secretion of gastrointestinal tract. InFraioli F, Isidori A, Mazzetti M (ed.). *Opioid Peptides in the Periphery*. Amsterdam: Elsevier Sciences Publisher, 1984: 243–247.
46 Suarez FL. Savaiano DA, Levitt MD. A comparison of symptoms after the consumption of milk or lactose-hydrolyzed milk by people with self-reported severe lactose intolerance. *N Engl J Med* 1995; **333**: 1–4.
47 Carroccio A, Mansueto P, Iacono G, Soresi M, D'Alcamo A, Cavataio F *et al*. Non-celiac wheat sensitivity diagnosed by double-blind

placebo-controlled challenge: exploring a new clinical entity. *Am J Gastroenterol* 2012; **107**: 1898–1906.
48 Volta U, Tovoli F, Cicola R, Parisi C, Fabbri A, Piscaglia M *et al*. Serological tests in gluten sensitività (nonceliac gluten intolerance). *J Clin Gastroenterol* 2012; **46**: 680–685.
49 Volta U. Indagine su sensibilità al glutine non celiaca: risultati preliminari. *Celiachia Notizie* 2013, **1**: 58–60.
50 Dezi R, Niveloni S, Sugai E, Pedreira S, Smecuol E, Vazquez H *et al*. Gluten sensitivity in the rectal mucosa of first-degree relatives of celiac disease patients. *Am J Gastroenterol* 1997; **92**: 1326–1330.
51 Bizzaro N, Tozzoli R, Villalta D, Fabris M, Tonutti E. Cutting-edge issues in celiac disease and in gluten intolerance. *Clin Rev Allerg Immunol* 2012; **42**: 279–287.
52 Volta U, Granito A, Parisi C, Fabbri A, Fiorini E, Piscaglia M *et al*. Deamidated gliadin peptide antibodies as a routine test for celiac disease: a prospective analysis. *J Clin Gastroenterol* 2010; **44**: 186–190.
53 Volta U, Corazza GR, Frisoni M, Valentini RA, Molinaro N, Bianchi FB *et al*. IgA antigliadin antibodies and persistence of jejunal lesions in adult celiac disease. *Digestion* 1990; **47**: 111–114.
54 Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a stndardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; **11**: 1185–1194.
55 Not T, Ziberna F, Vatta S, Quaglia S, Martelossi S, Villanacci V *et al*. Cryptic genetic gluten intolerance revealed by intestinal antitransglutaminase antibodies and response to gluten-free diet. *Gut* 2011; **60**: 1487–1493.