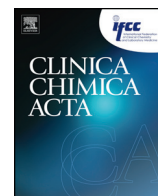




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Diagnostic accuracy of anti-gliadin antibodies in non celiac gluten sensitivity (NCGS) patients

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ABSTRACT

Background: Gluten is the target of several diseases such as wheat allergy (WA), celiac disease (CD) and non-celiac gluten sensitivity (NCGS). NCGS is a new clinical entity characterized by gastrointestinal and extraintestinal symptoms comparable to those of CD patients but to date still lacking of specific biomarkers so that NCGS diagnosis can be reached only by excluding CD and WA, and based on the direct association between gluten ingestion and symptoms onset.

Previous studies showed that anti-gliadin antibodies (AGA) IgG are the most prevalent positive antibodies in NCGS population.

Aim: The first aim of the study was to estimate AGA distribution and prevalence in a NCGS population. The second aim was to identify a serological pattern to help the diagnosis and/or to mark the NCGS disease.

Methods: Sera from 59 patients with suspected NCGS, 90 CD patients and 70 healthy individuals were assessed for AGA IgG/IgA, IgG/IgA deamidated gliadin peptide antibodies (DGP-AGA), tissue transglutaminase antibodies IgA (tTGA), endomysial antibodies IgA (EmA) and HLA typing (Eurospital, Trieste, Italy).

Results: We evaluated data by a dual statistical approach: logistic regression and receiver operating characteristic (ROC) analysis; therefore, we showed a poor diagnostic accuracy of AGA IgG in NCGS condition.

Conclusion: Our preliminary data showed that AGA IgG didn't seem to be a strongly sensitive marker, even if it has been recently proposed as promising marker for NCGS condition, together with negativity for other celiac disease related antibodies. It can partially help the NCGS diagnosis, if it is integrated in the overall management of the patient. More in-depth clinical and laboratory researches are mandatory.

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1. Introduction

Gluten is, by literature, a target of several diseases such as wheat allergy (WA), celiac disease (CD) and non-celiac gluten sensitivity (NCGS) [1,2].

NCGS is a new clinical condition characterized by intestinal (diarrhea, abdominal pain, bloating and flatulence) and extra-intestinal symptoms (such as headache, lethargy, attention-deficit, hyperactivity disorder, recurrent oral ulceration), with an early onset

after gluten ingestion, a rapid relief on a gluten free diet and an immediate relapse after the gluten challenge, related to immunological mechanisms still objects of several studies. The symptoms are not distinguishable from those of CD and WA [3–7].

NCGS epidemiological data are scarce: Sapone et al. [8] reported a 6% prevalence in a population of patients referred to Maryland Gastroenterology Clinics; these data could overestimate the real prevalence of the disease having been recorded in a referral center.

Biesiekierski et al. demonstrated, through a double blind study, that 30–40% of patients with inflammatory bowel disease diagnosed by Rome III criteria are NCGS patients [6].

A recent paper reported a possible prevalence of NCGS of 0.55% in the general population in the United States [9].

The diagnosis of NCGS is very difficult to date because of the lack of serological markers and specific clinical diagnostic criteria, so that NCGS diagnosis can be reached only by excluding CD and WA, and based on

Abbreviations: AGA, anti-gliadin antibodies; CD, celiac disease; DBPC, double blind placebo-controlled; DGP-AGA, deamidated gliadin peptide antibodies; EmA, endomysial antibodies; HI, healthy individuals; ROC, receiver operating characteristic; tTGA, tissue transglutaminase antibodies; WA, wheat allergy.

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the direct association between gluten ingestion and symptoms onset [8, 10,11,12]. The optimal diagnostic algorithm necessary to define the patient with NCGS is still under debate [13].

Recent studies demonstrated that a large part of NCGS patients have HLA typing and antibodies related to CD, mainly anti gliadin antibodies IgG [14–19] (Table 1).

The aim of the study was to estimate AGA distribution and prevalence in NCGS population trying to identify a serological pattern to help the diagnosis and/or to mark the NCGS disease.

A cutoff for AGA IgA and AGA IgG on NCGS patients, and not on CD population, was set up for better discriminating NCGS patients.

2. Material and methods

2.1. Patients

The study population included 59 patients attending the Referral Centre for Celiac Disease, Allergy and Clinical Immunology Unit, San Giovanni di Dio Hospital (Florence, Italy) between June 2011 and June 2012.

Of the 59 NCGS patients 40 were females (67.8%), with a female to male ratio of 2.1 to 1; the mean age was 45 ± 7.3 . These patients experienced both intestinal and extraintestinal symptoms after gluten ingestion, including abdominal pain, bowel abnormalities (either diarrhea or constipation), headache, fatigue, joint and muscle pain, leg or arm numbness, dermatitis (eczema or skin rash), depression and anemia, and all reported an improvement of symptoms on a gluten free diet and an immediate relapse after gluten challenge. They were considered gluten sensitive after the exclusion of CD and WA.

The control group consisted of 90 CD patients (mean age 41 ± 6.8 years; F/M 2.3) and 70 apparently healthy individuals (HI) (mean age 42 ± 9.1 years; F/M 2.8). (Table 2).

59 patients considered gluten sensitive fulfilled the following criteria characterizing gluten sensitivity:

- IBS-like syndrome defined according to the Rome II criteria [20].
- Negative serum for anti-transglutaminase (anti-tTG), anti-endomysium (EMA) IgA antibodies.
- Negative duodenal biopsy (Marsh 0–I).
- Negative for wheat allergy (specific IgE and Prick Tests) [21].
- Negative for anti-*Saccharomyces cerevisiae* antibodies (ASCA) and for anti-neutrophil cytoplasmic antibodies (ANCA).
- Resolution of symptoms in a gluten-free diet and their reappearance on double blind placebo-controlled (DBPC) wheat challenge.

2.2. Serological tests

2.2.1. Anti-tTG IgA test

Anti-tTG IgA antibodies were assayed in both CD and NCGS patients by Eu-tTG IgA (Eurospital, Trieste, Italy). Eu-tTG IgA is a sandwich type enzyme immunoassay. The human recombinant tissue transglutaminase enzyme is attached to microtitration wells. Antigen-specific antibodies in the patient's serum bind to the antigens. Non-specific antibodies are removed by washing. Horse-radish peroxidase labeled goat anti-human IgA are added and bind to human antibodies in the well. The excess conjugate

Table 2

Characteristics of the patients included in the study.

No. of patients with NCGS suspect diagnosis	59
Mean age, years $\pm$ SD	45 ± 7.3
Gender (M/F)	19/40
No. of patients with CD diagnosis	90
Mean age, years $\pm$ SD	41 ± 6.8
Gender (M/F)	27/63
Healthy individuals (HI)	70
Mean age, years $\pm$ SD	42 ± 9.1
Gender (M/F)	18/52

CD, celiac disease; NCGS, non-celiac gluten sensitivity; SD, standard deviation.

is washed away; then a chromogenic substrate is added. After an appropriate incubation period, the O.D. value is measured by means of a EIA reader (nm 450). The reagent kits came from the same lot in order to avoid lot variability. All of the calibrators were run in duplicate, and the specimens individually. The provided cutoff value of 16 U/mL was adopted.

2.2.2. EMA test

Indirect immunofluorescence test for the detection of EMA IgA antibodies against endomysial antigens on a section of monkey esophagus was performed on CD and NCGS patients by Antiendomysium kit (Eurospital, Trieste, Italy).

The Antiendomysium kit is used for the determination of serum IgA antibodies against endomysial antigens on a section of monkey esophagus. The antigen-antibody complex is visualized by a fluorescence microscope with the aid of a fluorescein-labeled antibody. Sera dilutions and operative procedure were done as indicated by the manufacturer [22,23].

2.2.3. AGA test

Both AGA IgA and IgG were assayed in all of patients: CD, NCGS and HI.

ELISA test α -gliatest IgA and IgG (Eurospital, Trieste, Italy) were employed, which uses purified α -gliadin as antigen. Gliadin peptides are attached to microtitration wells. Antigen-specific antibodies in the patient's serum bind to the antigens. Non-specific antibodies are removed by washing. Horse-radish peroxidase labeled goat anti-human IgA and IgG are added and bind to human antibodies in the well. The excess conjugate is washed away; then a chromogenic substrate is added. After an appropriate incubation period, the O.D. value is measured by means of a EIA reader (nm 450). The reagent kits came from the same lot in order to avoid lot variability. All of the calibrators were run in duplicate, and the specimens individually. Cutoff values, 50 U/mL for IgG and 15 U/mL for IgA, indicated by manufacturer were applied [24,25].

2.2.4. DGP-AGA test

All patients were checked for the presence of deamidated gliadin peptides antibodies IgG and IgA, by commercially available ELISA kits: α -glia PEP, IgA and IgG (Eurospital, Trieste, Italy).

The test uses a recombinant, synthetic, intact peptide, selectively deamidated as antigen, and provided cutoff values of 5.5 U/mL for IgA and 10 U/mL for IgG were adopted [26,27].

Table 1

AGA prevalences in previous studies.

Reference	NCGS patients (n.)	AGA IgG
Sapone et al. BMC Med. 2011 [16]	26	48%(AGA IgG/IgA)
Volta et al. J Clin Gastroenterol. 2012 [17]	78	56.4%
Carroccio et al. Am J Gastroenterol. 2012 [18]	276	55%
Volta et al. BMC Med. 2014 [19]	486	25%

AGA, anti-gliadin antibodies; AGA IgG/IgA, anti gliadin antibodies immunoglobulin A/immunoglobulin G; NCGS, non celiac gluten sensitivity.

Table 3

Antigliadin antibodies prevalence in NCGS, CD and HI populations at manufacturer's cutoff.

Prevalence	AGA IgG	AGA IgA	DGP IgG	DGP IgA
NCGS	6/59 (10.2%)	4/59 (6.8%)	3/59 (5%)	2/59 (3.4%)
CD	56/90 (62.2%)	63/90 (70%)	76/90 (84.4%)	80/90 (88.8%)
HI	2/70 (2.9%)	0/70	4/70 (5.7%)	2/70 (2.8%)

AGA, anti-gliadin antibodies; CD, celiac disease; DGP, deamidated gliadin peptide antibodies; HI, healthy individuals; NCGS, non-celiac gluten sensitivity.

2.2.5. Total serum IgA levels

In order to exclude IgA deficiency, serum IgA levels were assayed in all patients by BNII nephelometric analyzer (Siemens, Marburg, Germany).

2.2.6. Allergological tests

Skin prick tests to inhalant and to food allergens were performed in all patients [28]. Allergen specific IgE antibody levels for inhalant and food allergens were measured using Immuno CAP System (Phadia AB, Uppsala, Sweden). The detection limit of the assay was 0.10 kUA/l.

2.2.7. HLA typing

All patients were submitted to HLA class II typing for the DQ2 and DQ8 loci.

Genomic DNA was extracted and purified from blood samples collected in EDTA using the commercially available kit Eu-Gen Estrazione (Eurospital, Trieste, Italy).

Briefly, 200 μ l of blood sample were transferred to a 1.5 ml tube containing a 20 μ l of proteinase K. Then, 200 μ l of lysis solution were added to each sample, and the whole was thoroughly mixed on a vortex for 15 s. Following incubation at 56 °C for 10 min, the tube was shortly centrifuged, and 200 μ l ethanol (96–100%) was added to the sample that was mixed by vortexing for 15 s. Again, the tube was shortly

centrifuged and the content was transferred into a microfilter. Following a series of washing and centrifugation steps, purified DNA was finally eluted in 200 μ l of elution buffer.

The HLA-DQ2 and -DQ8 loci were typed by means of PCR amplification with sequence-specific primers (PCR-SSP) using the commercial kit Eu-Gen Risk (Eurospital, Trieste, Italy).

The kit contains primers designed to amplify specific sequences of the following alleles: DQA1*01, DQA1*02:01, DQA1*03, DQA1*05, DQA1*06, DQB1*02, DQB1*03:01, DQB1*03:02, DQB1*03:03, DQB1*03:04, DQB1*03:05, DQB1*04, DRB1*03, DRB1*04, DRB1*07, DRB1*11. Eight PCR reactions were carried out as multiplex amplifications for each sample. In addition to alleles, in each reaction test-tube, a specific genomic sequence codifying for human β -globin was also amplified. This gene acted as an internal control for all amplifications.

Amplification was carried out on an Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific Inc. Waltham, MA, USA) according to the manufacturer's instructions. The amplification protocol was as follows: 95 °C for 5 min, followed by 5 cycles at 95 °C for 30 s, 61 °C for 30 s and 72 °C for 45 s, then 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. The final incubation is at 72 °C for 7 min. The amplification products, obtained by means of the eight PCR reactions, were separated through "submarine electrophoresis" on precast, prestained 2.2% DNA FlashGel™ Cassettes, and were analyzed with the FlashGel™ System (Lonza Inc., Rockland, ME, USA). The identification of the molecular weights of the bands was carried out by comparing the migration distance of each band with that of the FlashGel™ DNA Marker (50 bp–1.5 kb) loaded into the gel upon each sample migration. Each run was documented with a camera, and haplotypes were defined by means of the dedicated software Easy-Gen (Eurospital, Trieste, Italy).

2.2.8. Duodenal biopsy

Endoscopy intestinal biopsy findings were available in all the NCGS patients. Four-six biopsies from the second duodenal portion were obtained in accordance with a standard diagnostic protocol

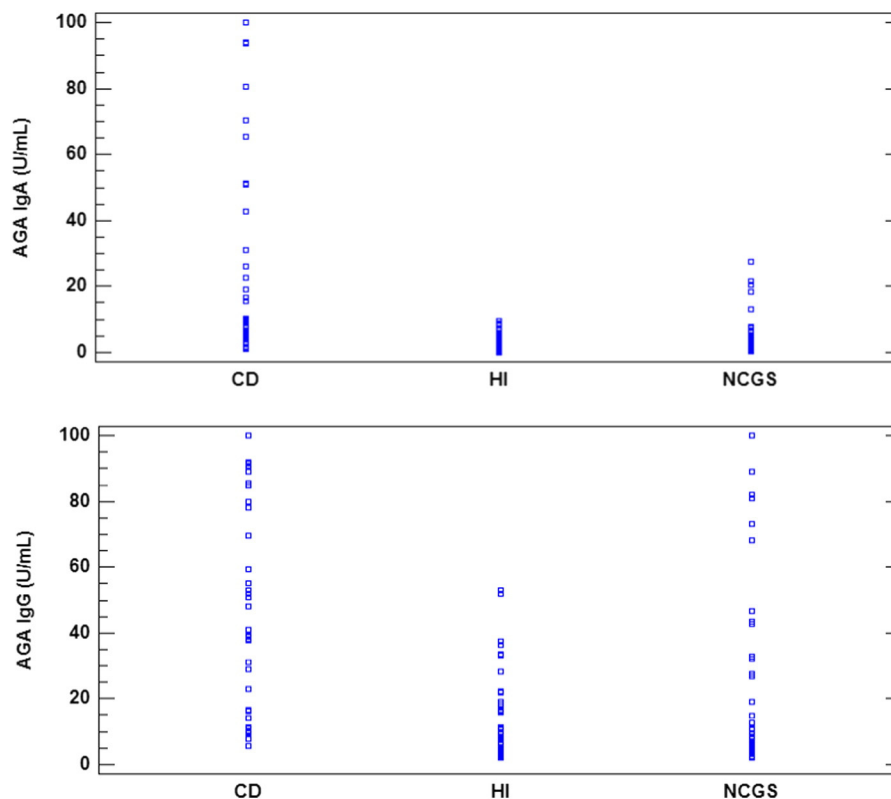


Fig. 1. AGA IgA and AGA IgG distributions in CD, HI and NCGS patients.

AGA, anti-gliadin antibodies; NCGS, non-celiac gluten sensitivity; CD, celiac disease; HI, healthy individuals.

Table 4
Study population: statistical data.

Population	N° patients	Average	Median	SD	STD skewness
AGA IgA (U/mL)					
NCGS	59	4.244	2.6	5.429	8.775
CD	90	35.16	9.65	39.424	2.705
HI	70	2.635	2.0	2.222	4.264
AGA IgG (U/mL)					
NCGS	59	17.868	6.9	24.561	6.421
CD	60	66.985	85.3	35.802	−1.581
HI	68	9.601	4.75	11.600	7.524

AGA, anti-gliadin antibodies; CD, celiac disease; HI, healthy individuals; NCGS, non-celiac gluten sensitivity; SD, standard deviation; STD, standard skewness.

and they were classified according to the Marsh–Oberhuber classification [1,29,30].

3. DBPCFC

Food challenge determines if an individual is in fact reactive to food. The double-blind, placebo-controlled food challenge (DBPCFC) is used for accurately diagnosing food allergies and for examining a wide variety of food related complaints. In this test, neither the physician nor the patient knows what the patient is being fed. For stopping an oral food challenge and declaring a challenge as positive or negative, symptoms should be objective and/or repetitive [31].

4. Statistical Analysis

4.1. Data analysis

Data analysis was performed by the software STATGRAPHICS XVI (StatPoint Technologies, Warrenton, VA). Receiver operating characteristic (ROC) analysis was carried out using the software MedCalc 14.8.1 (MedCalc software bvba, Ostend, Belgium).

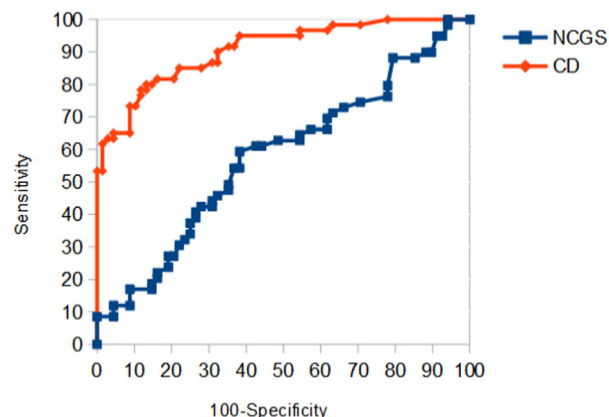
4.2. Distribution fitting

For each marker, data distribution was studied by standard skewness in order to verify the symmetry, then Gaussian distribution was verified by the Shapiro–Wilk W test. When Gaussian hypothesis was rejected we looked for the optimal distribution evaluating 37 different curves: first by the Anderson–Darling A^2 , then by Kolmogorov–Smirnov tests.

4.3. Logistic regression

The logistic regression statistic method measures the relationship among a categorical (dependent) variable Y and one or more

AGA IgA (AU)



AGA IgG (AU)

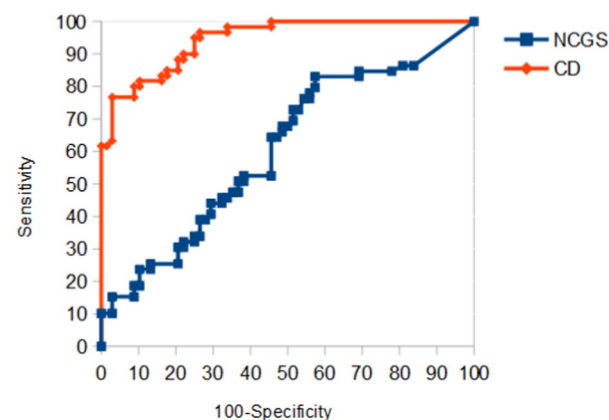


Fig. 3. ROC analysis for comparison of diagnostic performance of AGA IgA and AGA IgG in NSGD and CD conditions. AGA, anti-gliadin antibodies; NCGS, non-celiac gluten sensitivity; CD, celiac disease.

continuous (independent) variables x_i . It relates the probability of occurrence $P(x)$ of the outcome counted by Y to the independent variables x_i . An independent variable is defined as statistically significant when the P-value of its corresponding coefficient is ≤ 0.05 by the likelihood ratio test.

To determine whether the logistic regression model fits, several tests were performed.

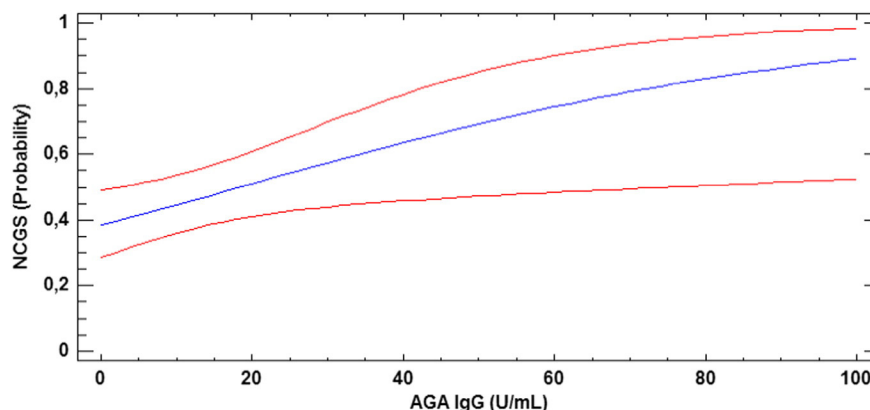
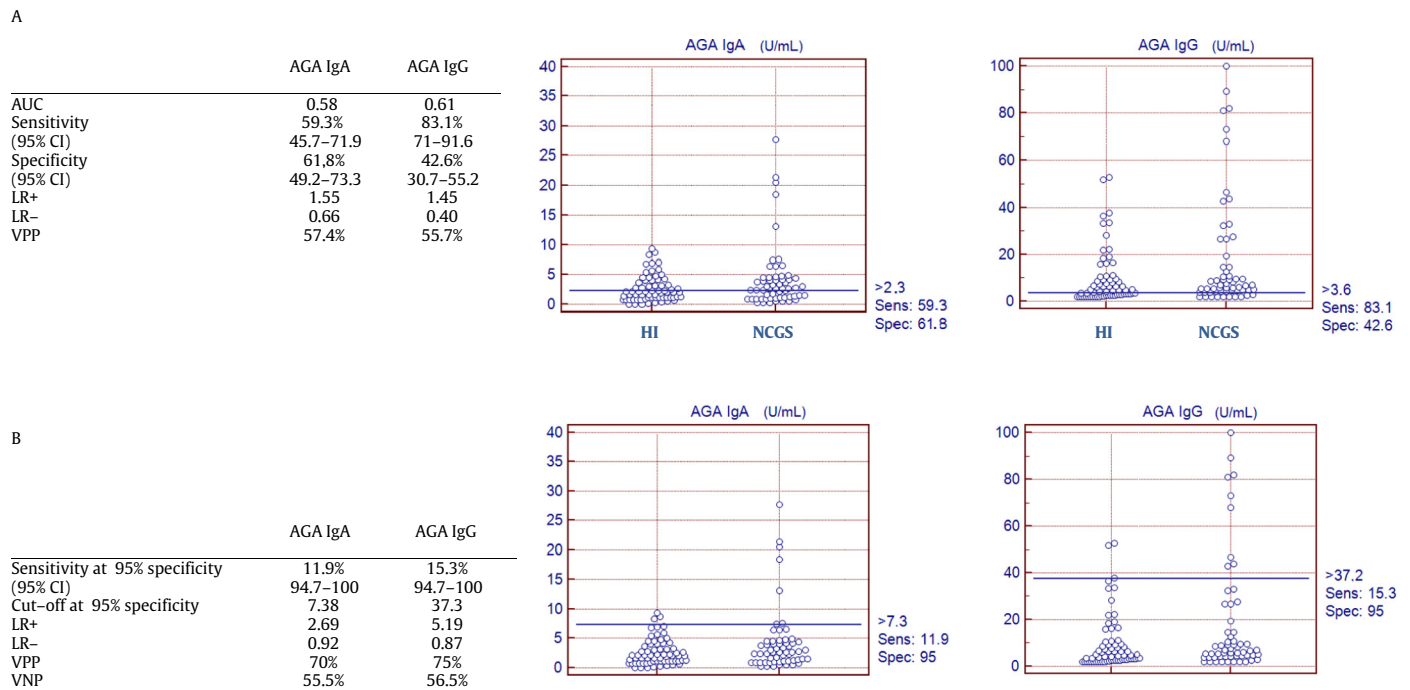


Fig. 2. Regression logistic model with 95% confidence limit. NCGS: disease probability (0 = absent; 1 = present). AGA, anti-gliadin antibodies; CD, celiac disease; DPG, deamidated gliadin peptide antibodies; HI, healthy individuals; NCGS, non-celiac gluten sensitivity.

Table 5

Diagnostic accuracy of AGA IgA and AGA IgG in NCGS condition determined by ROC analysis. (A) Sensitivity, specificity, LR +, LR –, NPV and PPV of AGA IgA and AGA IgG tests at the optimal predictive cutoff value. (B) Sensitivity, LR +, LR –, NPV, PPV and cutoff of AGA IgA and AGA IgG tests at 95% of specificity.



AUC: area under curve; LR +: positive likelihood ratio; LR –: negative likelihood ratio; NPV: negative predictive value; PPV: positive predictive value.

Final test is the percentage of deviance explained by the model: R^2 . Since it can range from 0 to 100%, R^2 is similar to R-squared statistic in multiple regression. An adjusted deviance R^2_{adj} is also computed because it considers the number of variables in the model.

For our data, the logistic regression analysis was performed by a stepwise method.

First, all variables are initially tested, then only the variables found to be statistically correlated were included in the model.

The general form of the best-fitting logistic regression model is $P = \exp(\eta) / [1 + \exp(\eta)]$

where:

P	probability
η	$k + a_1 * x_1 + a_2 * x_2 \dots a_n * x_n$
k	constant
a_n	slope for independent variable x_n .

4.4. Receiver operating characteristic (ROC) analysis

The performance parameters (sensitivity, specificity, positive likelihood ratio [LR +], negative likelihood ratio [LR –], positive predictive value [PPV], negative predictive value [NPV]) were calculated and ROC curves were drawn up.

Table 6

Autoantibodies prevalences (non celiac related).

Prevalence	ANA ($\geq 1:160$)	anti-ENA	anti-DNA	ASMA	AMA-LKM	APCA
NCGS	9/59 (15%)	2/59 (3.4%)	0/59	2/59 (3.4%)	1/59 (1.7%)	2/59 (3.4%)
CD	22/90 (24%)	3/90 (3.3%)	0/90	5/90 (5.5%)	1/90 (1.1%)	6/90 (6.6%)
HI	8/70 (11%)	1/70 (1.4%)	0/70	2/70 (2.8%)	0/70	3/70 (4.2%)

ANA, antinuclear antibodies; AMA, anti-mitochondrial antibodies; APCA, anti-parietal cell antibodies; ASMA, anti-smooth muscle antibodies; CD, celiac disease; ENA, anti-extractable nuclear antigen antibodies; HI, healthy individuals; LKM, anti-liver and kidney microsome antibodies; NCGS, non-celiac gluten sensitivity.

5. Results

Distribution of AGA IgA and IgG were compared in CD, HI and NCGS patients in order to identify a potential serological marker that could be helpful for NCGS diagnosis. At the manufacturer's cutoff AGA IgG antibodies were detected in 6/59 (10.2%) of NCGS population, in 56/90 (62.2%) of CD, and in 2/70 (2.9%) of HI. AGA IgA antibodies were present in 4/59 (6.8%) of NCGS, with respect to the 63/90 (70%) of CD patients and 0/70 assayed in HI control group. Furthermore, in the NCGS group only 3 patients showed a positivity to DGP IgG and 2 patients had DGP IgA with a prevalence respectively of 5% and 3.4%, similar to the prevalence of these antibodies in the healthy population (Table 3). In NCGS patients AGA IgG was the most frequent antibody found and titers were higher than HI population and as high as CD population. Comparing AGA IgA and AGA IgG distribution in HI and NCGS populations we observed a very similar spreading of the respective values with a wide overlapping of each marker in the two different populations with a lacking discrimination (Fig. 1).

Data are described by average, median, standard deviation, standard skewness, optimal distribution curve, logistic regression and ROC curve, using a 95% of confidence intervals. All variables examined appeared to be arranged in a non Gaussian distribution and the best fitting and most frequent was Log-logistic distribution. Only the CD data set of the AGA IgG showed a symmetric distribution (STD skewness <2) (Table 4). To

assess if a correlation between AGA IgA and IgG antibodies and NCGS occurs, a logistic regression statistic and ROC analysis were used as complementary approaches. Firstly AGA IgA and IgG were evaluated, then AGA IgA was considered non statistically significant marker and for this reason was excluded (P -value = 0.238). From logistic regression data, a statistically significant correlation between AGA IgG and NCGS was found.

The final model of the best-fitting logistic regression was:

$$\text{NCGS} = \exp(\text{eta}) / [1 + \exp(\text{eta})]$$

where

NCGS = probability of disease (0 = absent; 1 = present)

eta = $-0.474591 + 0.0257417 * \text{IgG AGA}$.

The percentage of deviance explained by the model (R^2) is 3.571% and the adjusted R^2 is 1.291%. Considering that the adjusted R^2 = 1.291 is low, the correlation was unsatisfactory from an application point of view, even if it was statistically significant (P -value = 0.0123) (Fig. 2). Evaluating data by ROC analysis, according to the first statistic approach, our results showed a poor performance of AGA IgA (area under curve = 0.578) and AGA IgG (area under curve = 0.608) antibodies in diagnostic accuracy of NCGS condition (Fig. 3). AGA IgA serum level > 2.3 U/ml (optimal predictive cutoff value calculated by ROC analysis) had a sensitivity of 59.3% and a specificity of 61.8%; AGA IgG serum level > 3.6 U/ml (optimal predictive cutoff value calculated by ROC analysis) had a sensitivity of 83.1% and a specificity of 42.6%. At a 95% specificity the sensitivities were 11.9% for AGA IgA and 15.3% for AGA IgG, showing both an inadequate percentages obtained (Table 5). Moreover at a 95% specificity PPV and NPV were respectively 57.4% and 62.9% for AGA IgA and 55.7% and 73% for AGA IgG. We performed 59 NCGS analyses of HLA-DQ variants and 10.4% resulted single copy DQ2 heterodimer in CIS (DQA1*05-DQB1*02/DQA1*X-DQB1*X), 23% carried a single copy of the DQB1*02 allele only, being DQA1*0201-DQB1*02/DQA1*X-DQB1*X, and 6.2% carried a double copy of the DQB1*02 allele, being DQA110201-DQB1*02/DQA1*0201-DQB1*02. Furthermore, 10.4% were DQ8 (DQA1*0301-DQB1*0302/DQA1*X-DQB1*X) positive. Given the association between celiac disease and other autoimmune diseases [32], the frequency of autoantibodies not involved in celiac disease [anti-nuclear antibodies (ANA), anti-extractable nuclear antigens (ENA), anti-DNA, anti-smooth muscle antibodies (ASMA), anti-gastric parietal cell antibodies (APCA), anti-mitochondrial (AMA), anti-liver-kidney microsomal antibodies (LKM)] was investigated in NCGS patients comparing to CD and HI populations. Statistically different frequencies of positivity among NCGS and CD and/or HI were not found; in this study, indeed, these autoantibodies were not significantly increased in patients with suspected NCGS ($P \geq 0.001$) (Table 6).

6. Discussion

NCGS has already been considered as a clinical entity after the increasing percentage of patients suffering from wheat sensitivity, complaining gastrointestinal and extraintestinal symptoms without meeting CD diagnostic criteria [33–38]. Nowadays, since specific biomarkers for NCGS have not been identified yet, the diagnosis is mainly an exclusion diagnosis based on clinical and histological findings.

Recent literature data showed that the difference between AGA IgG prevalence in NCGS condition and healthy individuals is relevant ranging from 25% to 56.4% [16–19]. Nevertheless, in our preliminary study the presence of AGA IgG alone, being negative other celiac related autoantibodies, is lower than in previous studies [16–19]. A possible explanation of the discrepancy among different studies could be the small number of patients included in some of them; thus determining a great variability on data obtained. Similarly our study included a limited number of patients for the difficulty of recruiting a large study

population; therefore additional studies are needed to assess the right role of the test in the NCGS diagnosis. One more reason of the low positivity percentage could be the different inclusion and exclusion criteria used in the studies; since the vast majority of them didn't use the DBPC challenge test, this could determine a larger number of patient enrolled. Moreover, the use of manufacturer's AGA IgG cutoff calculated on CD population for NCGS patients, could represent an important bias for the sensitivity and specificity calculated in the previous NCGS studies. Hence, we tried to identify and propose a new cutoff as an alternative to the CD specific one, to assess the AGA IgG role in NCGS condition. A combined statistical approach (logistic regression and ROC analysis) was used to assess the correlation of AGA IgG and NCGS condition; both statistical models showed a poor diagnostic accuracy of the marker in NCGS disease. At a 95% of specificity AGA IgG sensitivity was 15.3% with a PPV of 75% and a NPV of 56.5% showing an inadequate percentage obtained as potential strong NCGS marker. In fact only few patients (9/12) performing the test were positive with a high probability to be really affected (PPV = 75%); on the other hand, many patients (65/115) performing the test were negative with a low probability to be true positives (NPV = 56.5%). Moreover, AGA IgG distribution in the three different populations (NCGS, CD, HI) is characterized by a strong overlapping with a consequential lacking of discrimination. As NCGS is mainly an exclusion diagnosis and considering that many diseases are similar from a clinical point of view, it would be ideal to use AGA IgG as an exclusion test: this should mean a test with a high NPV in which patients, who are negative, should have a high probability to be really healthy and NCGS excluded. Indeed a serological marker, such as AGA IgG in NCGS condition, could be considered useful if it could well discriminate a disease population from a HI population or from other similar diseases population (disease control group).

HLA genes play the major role in determining susceptibility to gluten and half of the NCGS patients have displayed a slightly higher prevalence of HLA-DQ2 and/or HLA DQ8 molecules in their HLA system [8, 17, 39]. The genes encoding DQ2 or DQ8 molecules are present in 95% of the CD patients and the diagnosis of the disease is excluded in negative results for both HLA-DQ2 and HLA DQ8 in at least 95% (>95% negative predictive value). On the other hand healthy people present these genes as well (30%), but less frequently than in NCGS patients (50%) [8]. The data reported in the present article confirm a high prevalence (50%) of HLA DQ2 and HLA DQ8 in NCGS patients and no statistically significant differences were observed when comparing NCGS patients with IgG AGA antibodies positivity or negativity ($P > 0.001$) according to literature [17]. Moreover, the analysis of our results points out that NCGS patients display an elevated prevalence of HLA DQ2 and HLA DQ8 (50%) than that found in the general population and healthy blood donors [40]. Altogether, these results provide demonstration of specific genetic susceptibility in NCGS and this new condition is also genetically, as serologically, half way through CD and healthy population.

7. Conclusion

The correlation between AGA IgG and NCGS condition turned out to be slightly statistically significant. AGA IgG doesn't seem to be an adequately strong marker for its low statistical significance and for its lacking diagnostic accuracy; however it can partly help the NCGS diagnosis, integrated in the overall management of the patient.

Future researches are mandatory to identify reliable biomarkers for NCGS diagnosis and to better define the clinical and serological characteristics of NCGS patients.

Conflicts of interest

S.Catani and S.Ugolini are employees of Eurospital (Trieste, Italy). The other authors declare that they have no conflict of interest.

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