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Alimentary Tract

Anti-SARS-CoV-2 immunoglobulin profile in patients with celiac disease living in a high incidence area

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ABSTRACT

Background and aim: How symptoms and antibodies related to SARS-CoV-2 infection develop in patients with celiac disease (CD) is unclear. We aimed to investigate the impact of SARS-CoV-2 infection in CD patients.
Methods: CD patients were interviewed about the development of COVID-19 symptoms, compliance with anti-virus measures and adherence to a gluten-free diet (GFD). The presence of anti-SARS-CoV-2 IgG and IgA (anti-RBD and N proteins) was compared to that in non-CD subjects. Expression of the duodenal ACE2 receptor was investigated. When available, data on duodenal histology, anti-tissue transglutaminase IgA (tTGA), comorbidities and GFD adherence were analyzed.
Results: Of 362 CD patients, 42 (12%) reported COVID-19 symptoms and 21% of these symptomatic patients presented anti-SARS-CoV-2 Ig. Overall, 18% of CD patients showed anti-SARS-CoV-2 Ig versus 25% of controls ($p = 0.18$). CD patients had significantly lower levels of anti-N IgA. tTGA, duodenal atrophy, GFD adherence or other comorbidities did not influence symptoms and/or antibodies. The ACE2 receptor was detected in the non-atrophic duodenal mucosa of patients; atrophy was associated with lower expression of the ACE2 receptor.
Conclusion: CD patients have an anti-SARS-CoV-2 Ig profile similar to non-celiac controls, except for anti-N IgA. No risk factors were identified among CD parameters and GFD adherence.

1. Introduction

Since January 2020, the SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) pandemic has spread across the world and caused a huge number of infections and deaths [1].

The clinical picture of COVID-19 (coronavirus disease 2019) is variable, ranging from an asymptomatic course to severe pulmonary distress syndrome with high mortality rates. The most frequent COVID-19 symptoms are fever, cough and dyspnea but gastrointestinal symptoms have also been described [2].

It is still unclear which factors influence outcome in COVID-19 patients, but it seems that some comorbidities (e.g., hypertension or heart disease, overweight), tobacco use and male sex are risk factors; there is still doubt as to whether immunologi-

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cal/autoimmune disorders are also a risk factor, but the preliminary data are reassuring [3–7].

In accordance with WHO guidelines, a COVID-19 diagnosis is confirmed by a reverse transcription polymerase chain reaction (RT-PCR) test performed on samples taken from the respiratory tract (e.g., nasopharyngeal swabs); it should be noted that SARS-CoV-2 RNA can also be found in other specimens from COVID-19 patients, such as their stool [8].

SARS-CoV-2 enters human cells using the ACE2 (angiotensin converting enzyme 2) receptor, which is widely expressed on pneumocytes but also on other cells, such as enterocytes, thus explaining involvement of the gastrointestinal tract and, in particular, the small bowel (SB), which can also be affected by autoimmune disease [9,10].

Celiac disease (CD) is one of the most common autoimmune disorders, affecting approximately 1% of the general population. It is triggered by the ingestion of gluten-containing food in genetically susceptible subjects carrying the HLA DQ2 and/or DQ8 haplotypes. CD is characterized by the serological presence of autoantibodies (tissue transglutaminase antibody (tTGA), IgA and IgG), duodenal villous atrophy, increased intra-epithelial lymphocytes (IELs) and crypt hyperplasia. The mainstay of treatment of CD is adherence to a gluten-free diet (GFD), which generally leads to good control of symptoms, complete recovery of duodenal stricture and a good prognosis. In a small percentage of cases, CD can result in complications such as refractory celiac disease (RCD, subdivided into types I and II), ulcerative jejunoileitis, or enteropathy-associated T-cell lymphoma (EATL) [11,12].

Any interaction between SARS-CoV-2 infection and the immune system of CD subjects could be clinically and epidemiologically relevant, but there are limited data and few findings regarding this important issue [6,13]. With the present study, we aimed to investigate the co-occurrence of SARS-CoV-2 infection and COVID-19 in an Italian cohort of patients with CD living in an area (Milan) heavily affected by the pandemic.

2. Methods

2.1. Patients

Italian outpatient clinics were closed for all non-urgent practice from March 8th 2020 to May 4th 2020 due to the COVID-19 lockdown [14]. From April 20th 2020 to June 30th 2020, CD patients scheduled for an appointment during the March to May lockdown at the “Center for the Prevention and Diagnosis of Celiac Disease”, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico in Milan, were enrolled in a prospective monocentric study. Adult patients participated in a phone interview after they had given oral informed consent. The following data were collected: date of birth, sex, presence of COVID-19 symptoms (flu-like symptoms, fever or chills, cough, shortness of breath or difficulty breathing, fatigue, muscle or body aches, headache, recent loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, and diarrhea), the presence of a cohabitant with COVID-19, the taking of a SARS-CoV-2 PCR nasopharyngeal swab, compliance with anti-virus measures (shielding and social distancing scored on a numerical rate scale (NRS) from 0 to 10) and adherence to a GFD (NRS). When available within the last 12 months, duodenal histology findings (Marsh-Oberhuber classification) [15], anti-tissue transglutaminase IgA (tTGA) levels, presence of immunological comorbidities and adherence to a GFD (nutritional assessment and urine gluten peptide detection) were analyzed.

All patients also completed the ISMA (International Stress Management Association) Stress questionnaire, which has been validated for administration by phone [16]. The questionnaire was used to evaluate the susceptibility of subjects to stress on a 25-item scale. It presents dichotomous response options: ‘yes’ or ‘no’ with a ‘yes’ response corresponding to 1, while a ‘no’ response corresponds to 0. A total score of 4 or lower means that there is a low probability that the patient has a stress-related illness; a score of 5–13 points is associated with more stress-related health, mental or physical problems, for which counselling might be of help; and a score of 14 or more shows a higher probability of stress with unhealthy behaviors.

After lockdown ended, as people could now move outside the home, participants were invited to undergo a serological test for anti-SARS-CoV-2 antibodies from 1st June 2020 to 15th July 2020.

This study was approved by the local Ethics Committee (reference number 458_2020).

2.2. Antigen protein production

The recombinant Spike SARS-CoV-2 glycoprotein receptor binding domain (RBD) and the Nucleocapsid proteins were supplied by the COVID-19 laboratory of the European Institute of Oncology (IEO) by Drs Marina Mapelli and Sebastiano Pasqualato.

The RBD proteins were produced in mammalian HEK293F cells as glycosylated proteins by transient transfection with pCAGGS vectors generated in Professor Krammer’s laboratory [17]. The constructs were synthesized using the genomic sequence of the isolated virus, Wuhan-Hi-1, released in January 2020, and contain codons optimized for expression in mammalian cells. Secreted proteins were purified from the culture medium by affinity chromatography and quantified.

The recombinant nucleoprotein-N was produced in BL21-pLysS bacterial cells by pET28 overexpression vectors. The purification protocol consisted of a first affinity chromatographic column, followed by a size exclusion column.

Retrieved proteins were quantified, flash frozen in liquid nitrogen in aliquots and stored at −80 °C.

2.3. Detection of anti-SARS-CoV-2 antibodies by ELISA

The ELISA assay to detect immunoglobulins (Ig) G and A uses a fragment of the SARS-CoV-2 Spike glycoprotein (S protein) and the Nucleocapsid (N protein) as antigens based on the recently published protocol [17–19]. Briefly, after binding the proteins (RBD and N proteins) to a Nunc MaxiSorp ELISA plate, and blocking non-specific binding with PBS-BSA 3%, patient sera were applied to the plate to allow antibody binding at a final dilution of 1:200, revealed with secondary anti-human-IgG (BD, clone G18-145) and IgA (BioLegend, Poly24110) antibody conjugated to HRP. Samples were read on a GloMax reader at 450 nm. This ELISA test is not intended for commercial use and is currently under evaluation by Italy’s Ministry of Health (Aut.Min.Rich. 15/05/2020) for emergency use approval.

Positivity threshold levels were determined by ROC curves. Positivity for RBD was OD 0.29 for IgG and 0.5 for IgA. Positivity for Nucleocapsid was OD 0.32 for IgG and 0.38 for IgA.

A sex and age-matched group of 167 non-CD healthy subjects, tested in the same time frame as the CD patients, were used controls.

2.4. Histology and immunostaining

Ten duodenal samples from CD patients with atrophy and ten without were randomly selected for intestinal detection of the ACE2 receptor. ACE2 immunostaining (bs-1004R, Bioss Antibodies) was performed on endoscopic duodenal biopsies from CD patients using an automated immunostainer (Ventana BenchMark ULTRA, Roche Diagnostics) as previously described [20]. ACE2 staining was reported as the percentage of positive enteric cells (0–100%).

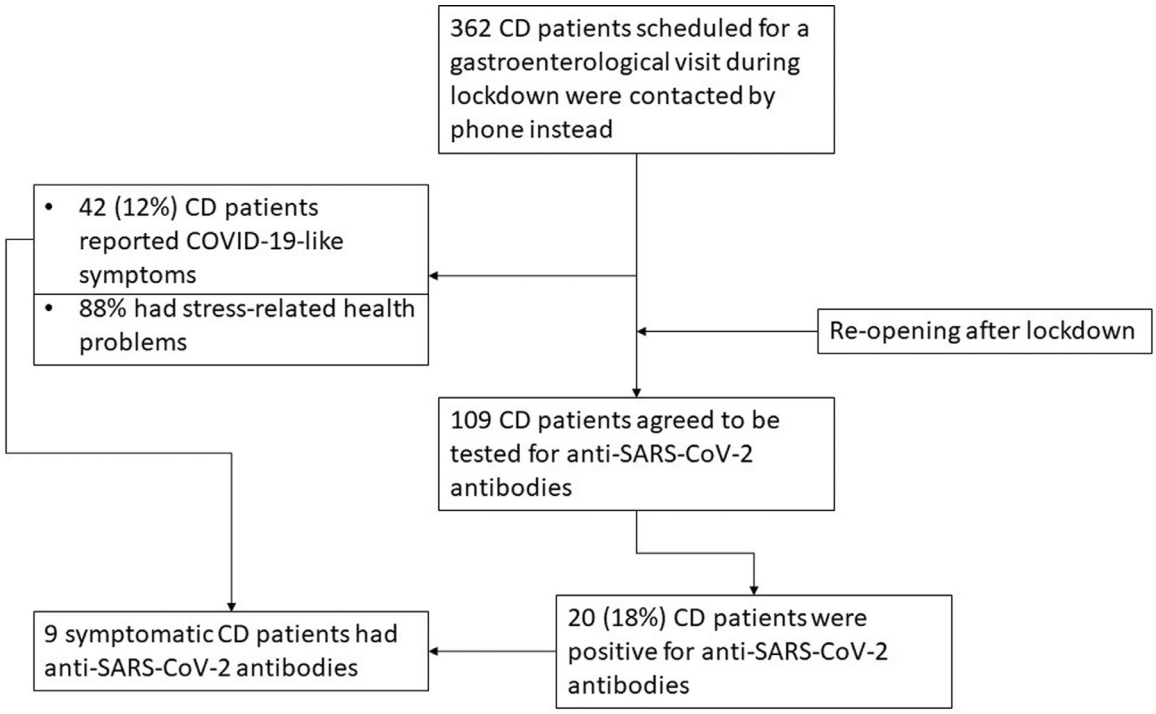

Fig. 1. Study flowchart. CD, celiac disease.

2.5. Statistical analysis

The data are described as mean ± SD or median (interquartile range) unless otherwise indicated. The continuous demographic variables were compared between the groups using an independent Student's *t*-test. Fisher's exact test and Yate's correction were used to evaluate the distribution of categorical variables. A 5% significance level was used. Variables found to have a statistically significant association in the univariate analysis were included in a multivariate backward stepwise logistic regression model. The software packages STATA®v. 13.1 (StataCorp LLC, College Station, TX, USA) and GraphPad Prism v. 6 (GraphPad Software, La Jolla, CA, USA) were used for analysis and graph processing.

3. Results

3.1. Patients and symptoms

A flowchart of the study is shown in Fig. 1. A total of 362 CD patients (288 (80%) females, age at enrolment 45±15 years, age at CD diagnosis 33±16 years) were spoken to by phone to evaluate the presence of COVID-19-like symptoms or a laboratory detected (i.e., nasopharyngeal swab) SARS-CoV-2 infection. Twenty-one (6%) patients had a diagnosis of refractory CD (RCD) according to international criteria. The following data were available: 23 (11%) of 202 (56%) patients tested for urinary gluten immunogenic peptides (uGIP) had gluten peptides in their urine, while tTGA serology results were available for 333 (91%) patients and positive in 78 (23%). Recent duodenal histology findings were available for 201 (55%) patients. A total of 42 (12%) patients reported symptoms potentially related to COVID-19 during the phone call. The percentages of reported symptoms are shown in Fig. 2, while the clinical and demographic data of these CD patients are reported in Table 1. There were no statistically significant differences between CD patients with and without COVID-19-like symptoms as regards age, age at CD diagnosis, sex, presence of tTGA or duodenal atrophy, GFD adherence or presence of other autoimmune disease. Only one patient had been hospitalized for mild respiratory distress without needing intensive care or respiratory support. Most patients reported strict compliance with antiviral measures (NRS 9.7 ± 0.6) and none reported difficulty adhering to a GFD (NRS 9.7 ± 0.9).

The ISMA Stress questionnaire demonstrated that 12% of patients had a low probability of having a stress-related illness (score 0–4), 73% reported stress-related health, mental or physical problems, for which they could benefit from counselling (score 5–13), and 15% showed a higher probability of experiencing stress with unhealthy behaviors (score 14–25). No demographic differences were observed between CD patients who did and did not have a stress-related illness.

3.2. Anti-SARS-CoV-2 antibodies

A total of 109 (31%) CD patients (88 females, age at enrolment 62±13 years, age at CD diagnosis 33±13 years) agreed to undergo a serological test for IgG and IgA against RBD and N proteins. At least one of the tested Ig was detected in 20 (18%) of these CD patients and in 42 (25%) control subjects ($p = 0.18$). In the group of patients testing positive for anti-SARS-CoV-2 Ig, 16 (80%) had anti-SARS-CoV-2 IgA, while 15 (75%) had IgG antibodies. In addition, 15 (75%) presented anti-RBD antibodies and 13 (65%) anti-N antibodies. The OD of the anti-SARS-CoV-2 immunoglobulins of CD patients and controls are detailed in Fig. 3; notably, CD patients showed significantly reduced values of anti-N IgA compared with otherwise healthy individuals.

Of the 20 CD patients with IgA and/or IgG in serum, 9/20 (45%) were referred for COVID-19-like symptoms. The clinical, serological and histological characteristics of the patients are reported in Table 2. Twenty-six (62%) of the 42 patients with COVID-19 symptoms underwent the serological test and 9/26 (34%) presented anti-SARS-CoV-2 Ig. No anti-SARS-CoV-2 Ig were detected in 17/26 (66%) tested patients who reported COVID-19-like symptoms. Symptoms reported in CD patients with or without anti-SARS-Cov-2 antibodies are reported in Fig. 3.

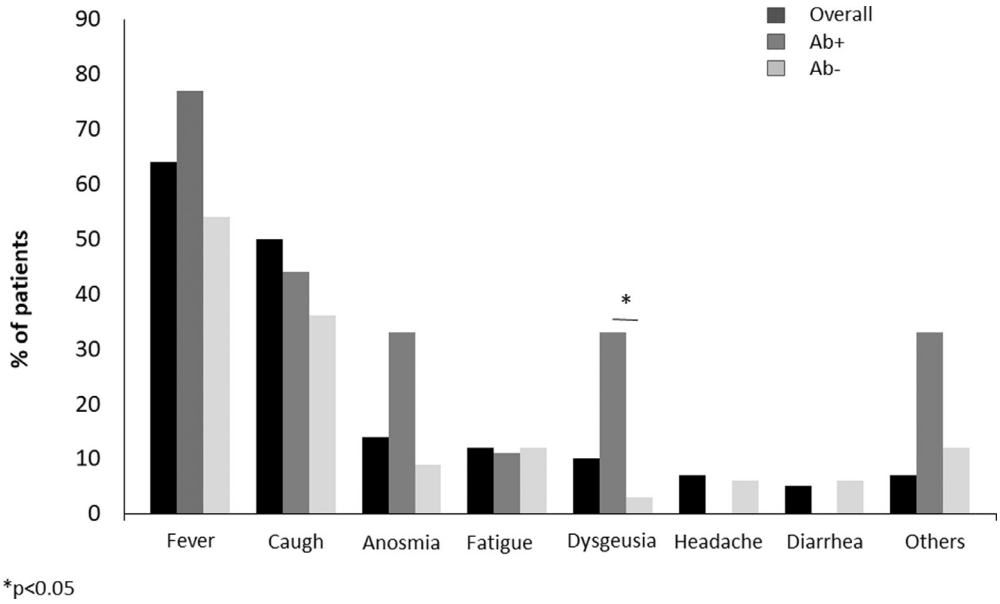

Fig. 2. COVID-19-like symptoms reported by celiac patients with (Ab+) and without (Ab-) anti SARS-CoV-2 Ig, during lockdown in the Milan area.

Table 1
Clinical and demographic characteristics of celiac patients with or without COVID-19-like symptoms.

	Overall $N = 362$	COVID-19 symptoms		
		Present $N = 42$	Absent $N = 320$	p Value (present vs absent)
Sex, female (%)	305 (79)	36 (85)	269 (84)	1.0
Age at enrolment (years)	45±15	41±13	46±15	0.05
Age at diagnosis (years)	32±15	30±13	34±16	0.13
BMI (kg/cm^2)	22.4 ± 3.4	22.2 ± 3.6	22.3 ± 3.9	0.96
Positive anti-tissue transglutaminase IgA (%)	23	23	21	0.69
Non-adherence to a GFD and/or positive urinary GIP (%)	10	10	11	1.0
Duodenal atrophy (Marsh 3a, 3b, 3c) (%)	64	59	67	0.47
Refractory celiac disease (%)	5	0	6	0.15
Presence of an AI comorbidity (%)	19	19	22	0.69

AI, autoimmune; BMI, body mass index; GFD, gluten-free diet; GIP, gluten immunogenic peptides.

Table 2
Clinical and demographic characteristics of celiac patients with or without anti-SARS-CoV-2 Ig.

	Anti-SARS-CoV-2 antibodies		
	Positive $N = 20$	Negative $N = 89$	p Value
Sex, female (%)	16 (80)	72 (81)	1.0
Age at enrolment (years)	47±13	44±15	0.54
Age at diagnosis (years)	34±17	33±16	0.80
BMI (kg/cm^2)	23.5 ± 5.8	22.4 ± 3.8	0.29
COVID-19-like symptoms, n (%)	9 (80)	17 (19)	0.02
Positive anti-transglutaminase IgA (%)	5 (25)	17 (19)	0.54
Non-adherence to GFD and/or positive urinary GIP (%)	0 (0)	5 (6)	0.58
Duodenal atrophy (Marsh 3a, 3b, 3c) (%)	7 (35)	29 (32)	1.0
Refractory celiac disease (%)	0 (0)	2 (2)	1.0
Presence of an AI comorbidity (%)	2 (10)	17 (19)	0.51

AI, autoimmune; BMI, body mass index; GFD, gluten-free diet; GIP, gluten immunogenic peptides.

CD patients with anti-SARS-CoV-2 Ig were asked to undergo a nasopharyngeal swab RT-PCR and one asymptomatic patient had a positive result.

3.3. Intestinal ACE2 receptor

Generally, the ACE2 receptor was present on the luminal surface of duodenal villi in CD patients with no signs of atrophy with an average percentage of positive cells of 55% (range 20–80%; Fig. 4A); conversely, when duodenal atrophy was present, ACE2 was weakly or not expressed on the enteric cells (Fig. 4B). Interestingly, we observed that ACE2 expression was more intense at the tip of the villi and less intense in the crypts. This result, although preliminary, may suggest that ACE2 expression has a gradient in the enteric mucosa along the villous-crypt axis.

All the analyzed duodenal samples are from CD patients with negative anti-SARS-CoV-2 serology not reporting a previous infection.

4. Discussion

The present study is the first attempt to investigate the effects of the SARS-CoV-2 pandemic on CD patients and their immunological response to viral infection. The serological anti-SARS-CoV-2

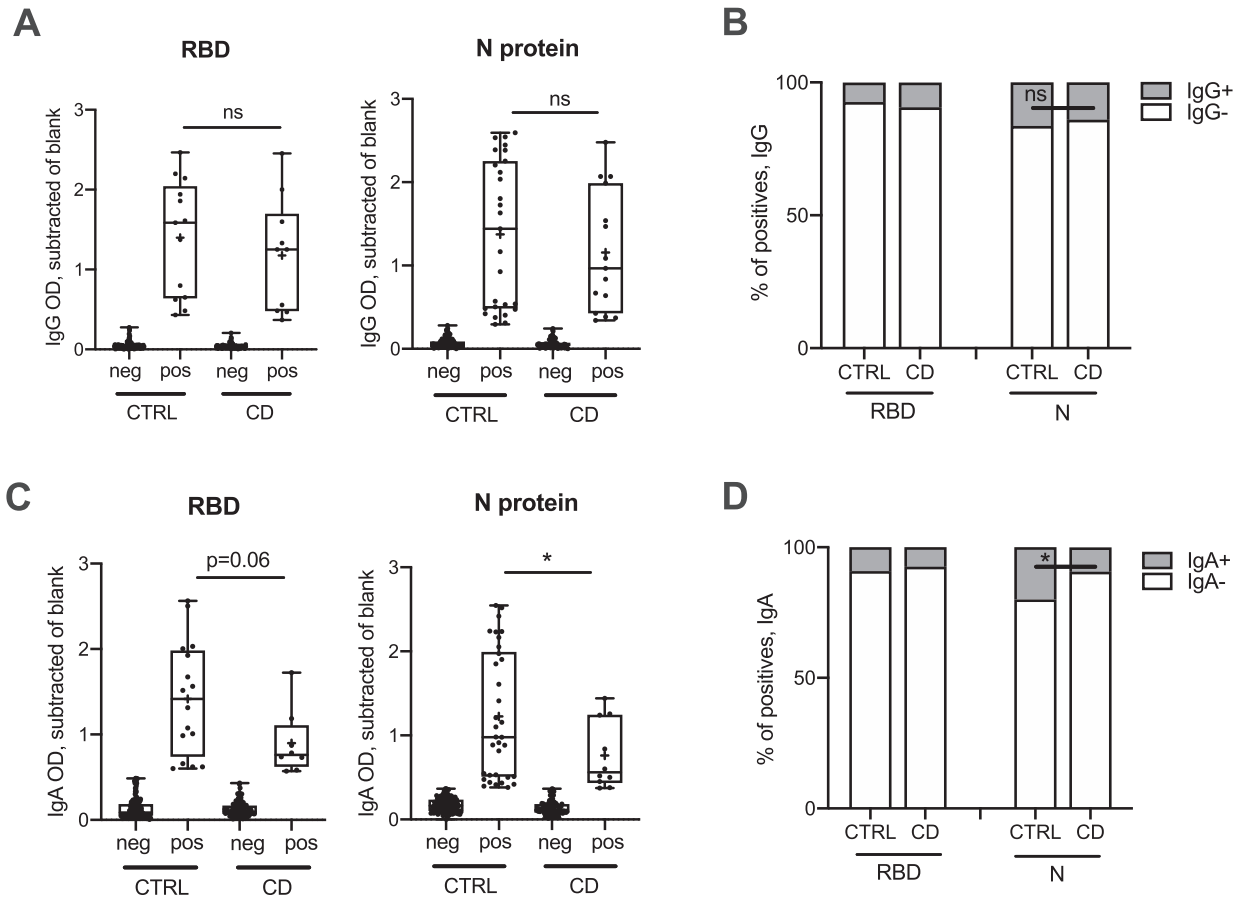

Fig. 3. SARS-CoV-2 specific antibody levels in Celiac disease (CD) individuals and healthy controls. (A,C) IgG (A) and IgA (C) levels in the sera of healthy subjects (CTRL, $n = 167$) and Celiac Disease patients (CD, $n = 109$) in ELISA assays (OD, optical density) against the RBD (left panels) and the N (right panels) SARS-CoV-2 viral proteins. (B,D) Frequencies of subjects tested positive for IgG (B) and IgA (D) antibody against RBD and N protein among healthy subjects (CTRL) and Celiac Disease (CD) patients . $p < 0.05$ (*) were regarded as statistically significant.

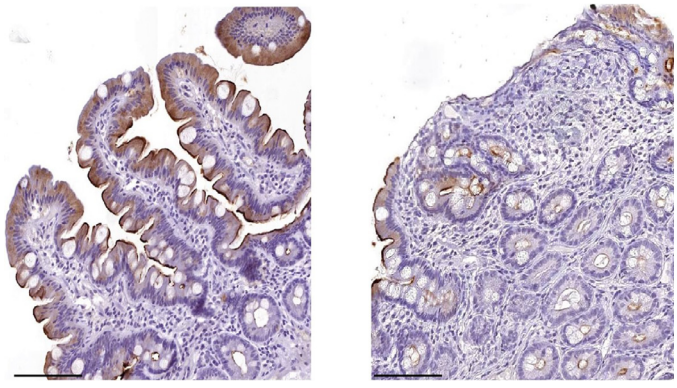

Fig. 4. Representative images of ACE2 expression in the non-atrophic (inactive; A) and an active (B) duodenal mucosa of a CD patient. Scale bar, 100 μm.

Ig profiles of CD patients are similar to those of non-CD patients. Moreover, the presence of CD biomarkers (tTGA, duodenal atrophy), autoimmune comorbidities and GFD adherence do not appear to influence the immune response in CD subjects.

In the investigated cohort of patients, COVID-19 symptoms were similar to those described in the literature, with a single case of hospitalization for mild respiratory distress. More than 80% of the CD patients had a stress-related illness and may need extra support because of the pandemic. Beside CD, the reported rate could be related to the high incidence of COVID-19 in the studied region and the high percentage of female sex in the cohort of patients [21,22].

The present findings are of particular relevance as they were obtained from an area badly affected by the SARS-CoV-2 pandemic, with all patients living in the Milan–Bergamo area of Northern Italy [21] and 20% testing positive for anti-SARS-CoV-2 Ig. Half of patients with positive anti-SARS-CoV-2 serology reported symptoms, while others did not have any symptoms, suggesting asymptomatic disease. The presence of symptomatic patients with negative for anti-SARS-CoV-2 Ig suggests that other factors could be involved in the patients' clinical picture. Unfortunately, during the first lockdown, very few nasopharyngeal swabs were taken, including in hospitals, due to a shortage of resources and, therefore, PCR results are not available to confirm or refute the findings [21]. However, serological testing has been used in recent months to monitor viral spread in the general population and among individuals at increased risk of contracting COVID-19, including immunosuppressed patients [23]. Here, we used a test obtained from the laboratory of Professor Krammer [17,18] that had received FDA approval [17–19]. The assay showed excellent specificity and sensitivity [19,23].

Interesting, the IgA response against SARS-CoV-2 has been reported to be possibly associated with a mucosal immune response in the gut and lungs [24]. A recent paper suggested that IgA production might occur locally at mucosal sites, possibly correlating with viral load, the duration of viral exposure and virus entry route [25]. In line with this suggestion, it has been shown recently that the highest levels of IgG and IgA antibodies against the Spike S1

domain were associated with severe disease [26]. IgA production correlates with our findings in the CD duodenal mucosa where we have found different levels of the ACE2 receptor. We reported the first findings in CD, but it was previously demonstrated that all components of the renin-angiotensin system (RAS) are present in the small bowel mucosa [27]; the fact that ACE2 was less present in the crypts of the enteric mucosa than at the villi tips might explain its modest expression in the mucosa of CD patients with flattened mucosa. Probably, a way by which SARS-CoV-2 elicits inflammation in the gastrointestinal tract is related to the decrease in mucosal ACE2 presence after virus entry into enteric cells [28].

The genetic background of CD is well known and strongly linked to HLA DQ2-DQ8 haplotypes. Several papers have focused on the identification of possible predisposing genetic factors for either the development of COVID-19 [29,30] or more severe clinical involvement [31,32]; However, none of the studies identified a significant association with the DQ2 haplotype. Although we did not analyzed the genetic background, this could be in agreement with the severity of COVID-19 infection observed in the CD cohort described here, only one of whom required hospitalization.

Although deficiencies of both fat- and water-soluble vitamins have been documented in patients with CD, the investigated patients did not report any difficulty in following a GFD during lockdown [33]. This could be an important factor, as previous research on other viral infections has indicated that nutritional status plays a significant role in patient outcome [34,35].

Notably, none of the routinely evaluated CD biomarkers (tTGA, duodenal atrophy), GFD adherence or the presence of autoimmune comorbities influenced symptoms and/or Ig response.

The results are significant but there are several limitations, including the lack of PCR tests due to a shortage of resources, the impossibility of seeing patients in person and, after lockdown, the restrictions on movement and difficulty completing anti-SARS-CoV-2 serology tests, which could have influenced findings.

In conclusion, based on our results and considering the study limitations, we have found the serological anti-SARS-CoV-2 response in CD patients was similar to that seen in healthy controls, suggesting also a positive response to vaccines, although dedicated studies are needed [36]. Moreover, CD patients reported optimal adherence to a GFD and compliance with anti-viral measures. However, these patients have increased levels of stress, suggesting the provision of telehealth services would be appropriate [37].

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Declaration of Competing Interest

None declared.

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