Seronegative Villous Atrophy in Children: Clinical and Immunohistochemical Features

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ABSTRACT

Objectives: Villous atrophy (VA) is not pathognomonic of celiac disease (CD). We aimed at reporting distribution, clinical, and immunohistochemical features of seronegative VA (SNVA) in a pediatric population.

Methods: We retrospectively collected data from patients who underwent intestinal biopsies between 2010 and 2017 and showed VA without serum CD-associated autoantibodies. Marsh-Oberhuber grading was used. Density of intraepithelial lymphocytes (IELs) expressing CD3 or TCRγδ+ receptor and of *lamina propria* CD25+ cells was assessed by immunohistochemistry. Intestinal deposits of anti-tissue tranglutaminase2 (anti-TG2) were also investigated by double immunofluorescence.

Results: Over a 7-year period, 64 out of 1282 patients with VA had negative serum CD serology. Diagnoses were: inflammatory bowel diseases (IBD) (21/64), Gastro-Esophageal Reflux Disease (GERD) (12/64), food allergy (8/64), infections (7/64, of which 3 HIV infections), immune deficiency (3/64), short bowel syndrome (3/64), congenital diarrhea (2/64), other/inconclusive diagnosis (8/64). Forty-four, 15, and 5 showed Marsh 3a, 3b, and 3c lesion, respectively. The latter category included 2 patients with Crohn disease, 2 with immunodeficiencies, 1 with lymphohistiocytosis. In 41/46 (89%) patients, mononuclear CD25+ cells were above the cut-off, indicating mucosal inflammation but only 18/46 (39%) had IELs and TCRγδ + IELs above limits of normality. In 10 of 46 (22%) patients, a positive immunofluorescence indicated the presence of anti-TG2 mucosal antibodies.

Conclusions: SNVA is not rare representing up to 5% of the cases of VA. Most patients have a Marsh 3a lesion. Immunohistochemical analysis may be helpful in excluding CD, whereas the finding of mucosal anti-TG2, particularly with a weak staining, shows no absolute specificity for CD.

Key Words: γδ intraepithelial T cells, anti-TG2 antibodies, enteropathy, nonceliac villous atrophy, seronegative celiac disease, seronegative villous atrophy

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What Is Known

- Celiac disease is the most common cause of intestinal villous atrophy.
- Seronegative villous atrophy is considered rare in pediatric age; few data are available in literature.

What Is New

- Seronegative villous atrophy represents up to 5% of the cases of villous atrophy in children.
- Most frequent causes of seronegative villous atrophy are inflammatory bowel diseases, gastroesophageal reflux disease, food allergy, infections, immune deficiency, short bowel syndrome, and congenital diarrhea.
- Seronegative celiac disease is virtually absent in children.
- Lamina propria is often inflamed, but lymphocytic infiltration of the epithelium is present in less than half of the cases of seronegative villous atrophy.

Normal duodenal mucosa has numerous finger-like projections or villi and an equal number of crypts (1). Villi length to crypts depth ratio is typically more than 2 to 3 in normal conditions. In case of mucosal damage, several morphological changes can be observed, leading at the end to crypts hyperplasia and villous atrophy (VA) (2). Michael N. Marsh first described the progression of the intestinal damage in celiac patients after gluten exposure. Marsh classification ranges from a normal mucosa (pre-infiltrative lesion, type 0), to a completely destroyed mucosa (hypoplastic lesion, type 4) (3). As stated by Marsh himself, flattening does not involve attrition of every villous to the level of the crypts openings. More than a simple ''atrophic process,'' it is a complex tissue remodeling including expansion of intervillous ridges, mesenchyme, and crypts (4).

More than 90% of VA is attributed to celiac disease (CD), especially if associated to a positive serology, regardless of patients' age (5). An obvious exception is neonatal age, when gluten has still not been introduced in the diet and VA is more frequently related to a congenital disorder. The dilemma occurs when VA appears in the context of a negative CD serology. This clinical entity is termed as seronegative villous atrophy (SNVA) and is generally affected in adults by a worse prognosis compared with classical seropositive CD (mortality 6% people/year vs 0.2% people/year) (6).

Recently, this condition has been studied in the adult population. The single most frequent cause of SNVA is represented by seronegative celiac disease (SNCD): from 7 to 45% according to different studies [31% Aziz et al (7), 45% Volta et al (8), 7% Schiepatti et al (9)]. A small percentage of celiac patients in fact display VA but are negative to specific serology. Diagnosis of this condition strictly relies on the histological response to a gluten-free diet (GFD), after other rare forms of enteropathy unrelated to gluten ingestion have been ruled out. Surprisingly, SNVA is typically

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characterized by a more severe degree of VA and clinical presentation and generally has a later onset in age compared with seropositive CD. Other causes of SNVA include infections, congenital or acquired immunodeficiencies, bacterial overgrowth syndrome, inflammatory bowel disease (IBDs), drugs (NSAID, olmesartan, immunosuppressors), autoimmune enteropathies and food allergies, with variable frequencies depending on the authors (10). There are, however, few studies regarding SNVA in children.

The aim of our work was to describe the clinical presentation, histological, and immunohistochemical features of duodenal biopsies in a cohort of pediatric patients with SNVA.

METHODS

Patients

Over a 7-year period (from 2010 to 2017), we retrospectively collected data from all the children with intestinal villous atrophy but without CD-associated antibodies diagnosed in our center, University Federico II, Naples. The identification of SNVA was based upon duodenal biopsies showing villous atrophy and negative CD serology. Reasons that led to the execution of an EGDS and a duodenal biopsy in these patients were failure to thrive, chronic diarrhea, melena, unresponsive iron deficient anemia, upper abdominal pain and heartburn. In all the cases, duodenal biopsies were performed regardless of the presence of duodenal macroscopic lesions. For each patient age at diagnosis, clinical symptoms and final diagnosis were recorded. This study was approved by the ethical committee of the University Federico II, Naples (number 58/20).

Celiac Serology

All patients were tested for total serum IgA and twice for anti-tissue transglutaminase (anti-TG) IgA and anti-endomysium (EMA). In case of IgA deficiency, IgG antibodies were evaluated in order to exclude CD. To measure serum anti-TG antibodies, an enzyme-linked immunosorbent assay kit was used, based on a human recombinant antigen (Eu-tTg IgA, Eurospital, Trieste, Italy)

Duodenal Biopsy and Immunohistochemical Analysis

In each patient, esophagogastroduodenoscopy with 5 biopsies (1 from the bulb and 4 from the distal duodenum) was carried out. According to our hospital protocol, 4 of 5 fragments, including 1 from the bulb, were fixed in 10% formalin, embedded in paraffin, and then stained with hematoxylin-eosin. The histological and morphometrical analysis by light microscopy was performed by 2 experienced pathologists. No double check was performed. Only correctly oriented samples were evaluated. Not correctly oriented specimens were recut in order to make them readable. A villous height: crypts depth ratio ≥ 2 was considered normal (11). Among biopsies with a normal villous height: crypts depth ratio, Marsh 0 was defined by the presence of less than 25 intraepithelial lymphocytes (IELs) per 100 enterocytes and Marsh 1 by the presence of 25 or more intraepithelial lymphocytes (IELs) per 100 enterocytes. Marsh score was assigned based on the fragment with the most severe lesion.

The evaluation of these 4 fragments was made blinded to any serology results. One fragment (not from the bulb) was put in an optimal cutting temperature compound (Killik, Bio-Optica, Milan, Italy), stored in liquid nitrogen, and used for immunohistochemical staining for CD3+, TCRγδ+, and CD25+ cells,. The number of stained cells per millimeter of epithelium determined the density of cells expressing CD3 and TCRγδ in the intraepithelial compartment. Cutoff values for CD3+ and TCRγδ+ cells were 34 and 3.4 mm per epithelium, respectively. On the other hand, the number of cells expressing CD25 in the lamina propria was evaluated within a total area of 1 mm^2. The usual cutoff value for CD25+ cells is 4 mm^2 lamina propria. To determine the cutoff values to be used, 100 children with untreated CD and 50 non-CD control children were studied. Percentiles were obtained using the SPSS software (IBM, Chicago, IL). Cutoff values represented the 90th percentile of non-CD patients.

Intestinal Deposits of Anti-TG2 IgA Antibodies

Duodenal biopsies were also investigated for the presence of extracellular deposits of anti-TG2 IgA antibodies as previously described (12). The evaluation of the deposits, performed considering the pattern and the intensity of the staining, was graded semiquantitatively as follows: negative, very weak, weak with patchy distribution, strong with patchy distribution, and strong with a homogenous distribution.

RESULTS

Villous Atrophy Etiology and Histology Analysis

Over a 7-year period, between 2010 and 2017, VA was found in 1282 patients. One thousand two hundred eighteen out 1282 had a

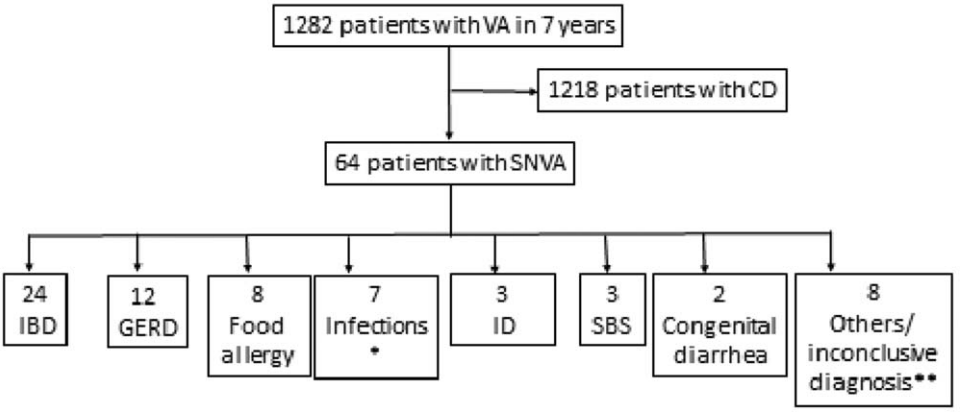

FIGURE 1. Patients enrolled in the study. CD = celiac disease; IBD = inflammatory bowel disease; GERD = gastro-esophageal reflux disease; ID = immune deficiency; SBS = short bowel syndrome. *Seven infections that included 3 HIV patients. ** Included cases of Down syndrome, lymphohistiocytosis, duodenal membrane, and duodenal fistula, ectodermic dysplasia, 2 inconclusive diagnosis.

TABLE 1. Clinical, serological, and immunohistochemical features of patients affected of seronegative villous atrophy

Patient No.	Sex	Diagnosis group	Age at diagnosis	CD serology	Marsh stage	V/C	CD3	γδ	CD25	Anti-tg staining	Notes
1	M	Congenital diarrhea	1	neg	M3b	0.9	3.2	0.8	36	Absent	Tufting enteropathy
2	M	Congenital diarrhea	0.6	neg	M3b	1.1	n.e	n.e	n.e	n.e	Unknown origin*
3	M	Food allergy	2.6	neg	M3a	1.8	46	6.6	8	Absent	Eosinophilic esofagitis
4	F	Food allergy	0.5	neg	M3a	1.9	n.e	n.e	n.e	n.e	Cow's milk protein allergy
5	F	food allergy	1.1	neg	M3a	1.7	35	4.5	34	Absent	Cow's milk protein allergy
6	F	Food allergy	7.9	neg	M3a	1.3	5	0.4	14	Absent	Eosinophilic gastroenteritis
7	F	Food allergy	10.9	neg	M3a	1.8	28	2	38	n.e	Cow's milk protein allergy
8	M	Food allergy	0.7	neg	M3a	1.8	24	3.5	5	Absent	Cow's milk protein allergy
9	F	Food allergy	1.1	neg	M3a	1.8	31	2	7	Absent	Cow's milk protein allergy
10	M	Food allergy	5.4	neg	M3a	2.0	42.4	1.2	61	n.e	Cow's milk protein allergy
11	M	GERD	2.7	neg	M3a	1.6	40	4.1	17	Absent	
12	F	GERD	14.4	neg	M3a	1.7	33.2	4.8	18	Weak	
13	M	GERD	6.5	neg	M3a	1.8	27	1.4	23	Absent	
14	F	GERD	10.0	neg	M3a	1.6	23.4	1.1	15	Weak	
15	M	GERD	12.3	neg	M3a	1.6	17	2.8	11	Weak	
16	F	GERD	4.8	neg	M3a	1.8	86	2.5	26	Absent	
17	M	GERD	2.4	neg	M3b	1.2	n.e	n.e	n.e	n.e	
18	F	GERD	12.0	neg	M3b	1.6	26	1.2	21	Absent	
19	M	GERD	1.5	neg	M3a	1.7	8.2	2	112	Weak	
20	M	GERD	7	neg	M3a	2.0	53.3	2.5	79	Absent	
21	F	GERD	12.6	neg	M3a	1.8	21.7	1.6	13	Absent	
22	M	GERD	8.8	neg	M3a	1.7	34.75	2.8	14	Absent	
23	F	IBD	10.3	neg	M3c	0.8	28	0.44	16	Absent	Crohn
24	F	IBD	13.4	neg	M3b	1.4	n.e	n.e	n.e	n.e	Crohn
25	F	IBD	6.7	neg	M3a	1.7	n.e	n.e	n.e	n.e	UC
26	M	IBD	13.6	neg	M3a	1.8	n.e	n.e	n.e	Weak	UC
27	F	IBD	13.5	neg	M3a	1.8	28	0.8	19	Absent	Crohn
28	M	IBD	10.7	neg	M3a	2.0	26	1.3	10	Absent	UC
29	F	IBD	17.7	neg	M3a	2.0	25.2	6.6	5	Weak	Crohn
30	M	IBD	11.2	neg	M3a	1.7	75	0.36	8	Absent	Crohn
31	M	IBD	15.9	neg	M3b	1.2	34	3.2	10	Weak	UC
32	M	IBD	15.9	neg	M3c	n.e.	24	0.8	30	Absent	Crohn
33	F	IBD	11.7	neg	M3a	1.9	20	0.36	26	Absent	UC
34	M	IBD	13.3	neg	M3a	1.6	n.e	n.e	n.e	n.e	UC
35	M	IBD	16	neg	M3b	1.5	n.e	n.e	n.e	n.e	UC
36	F	IBD	8.2	neg	M3a	1.8	36	1.9	11	Absent	Crohn
37	F	IBD	16.4	neg	M3a	1.6	n.e	n.e	n.e	n.e	Crohn
38	F	IBD	25.5	neg	M3a	2.0	10.7	2	4	Absent	UC
39	M	IBD	13.5	neg	M3a	1.6	19.8	1	28	Weak	UC
40	F	IBD	13.6	neg	M3b	1.2	16.4	2.1	35	Absent	Crohn
41	M	IBD	12.5	neg	M3a	2.1	7.5	1.9	62	Patchy	Crohn
42	F	IBD	12.6	neg	M3a	2.1	11.6	1.9	68	Absent	UC
43	F	IBD	12.9	neg	M3a	1.6	27	2.1	14	Absent	UC
44	M	Immunodeficit	2.3	neg	M3c	0.8	n.e	n.e	n.e	n.e†	IgA deficiency
45	M	Immunodeficit	0.6	neg	M3a	1.0	n.e	n.e	n.e	n.e	SCID
46	M	Immunodeficit	1.1	neg	M3c	n.e.	n.e	n.e	n.e	n.e	Chronic granulomatous disease

Patient No.	Sex	Diagnosis group	Age at diagnosis	CD serology	Marsh stage	V/C	CD3	γδ	CD25	Anti-tg staining	Notes
47	M	Infections	15.8	neg	M3a	1.6	14	0.6	7	Absent	Hp infection
48	M	Infections	11.1	neg	M3a	2.0	7.6	1.8	11	Absent	Yersinia infection
49	F	infections	10.8	neg	M3a	2.3	n.e	n.e	n.e	n.e	Hp infection
50	M	Infections	0.3	neg	M3a	1.8	n.e	n.e	n.e	n.e	HIV
51	M	Infections	2.8	neg	M3b	1.2	15.3	5	47	Absent	SIBO
52	F	Infections	17	neg	M3b	1.5	37.6	3.8	18	Absent	HIV
53	M	Infections	9.1	neg	M3a	2.3	n.e	n.e	n.e	n.e	HIV
54	F	Other	3.2	neg	M3a	1.8	67	1.4	n.e	Absent	Down syndrome
55	M	Other	0.5	neg	M3b	1.0	n.e	n.e	n.e	n.e	ectodermal dysplasia
56	F	Other	0.2	neg	M3b	1.1	14.6	3.6	74	Absent	duodenal membrane
57	M	Other	0.2	neg	M3c	0.6	17	2.1	42	Absent	lymphohistiocytosis
58	M	Other	0.2	neg	M3b	1.0	n.e	n.e	n.e	n.e	Down syndrome
59	F	Other	0.3	neg	M3a	2.0	13	1.8	24	Absent	Undiagnosed*
60	M	Other	0.5	neg	M3b	1.1	25.5	6.5	94	Absent	Melena of unknown origin*
61	F	Other	1.7	neg	M3a	n.e.	29.3	3.6	2	Absent	Duodenal fistula
62	M	SBS	0.4	neg	M3a	1.8	30	10.8	38	Absent	Postsurgery
63	F	SBS	5.3	neg	M3b	2.6	12.7	0.8	11	Weak	Postsurgery
64	M	SBS	1.8	neg	M3a	1.5	n.e	n.e	n.e	n.e	Postsurgery

GERD = gastroesophageal reflux disease; IBD = inflammatory bowel disease; n.e = not evaluated; SBS = short bowel syndrome; SCID = severe combined immunodeficiency; UC = ulcerative colitis.
*Diagnosis made before gluten introduction.
†Absence of IgM anti-tissue transglutaminase deposits in the intestinal mucosa.

positive CD serology whereas 64/1282 (5%) were defined as SNVA (55% boys, mean age at diagnosis: 5.9 years). Clinical diagnoses were: inflammatory bowel diseases (IBD) (21/64), gastro-esophageal reflux disease (GERD) (12/64), food allergies (8/64), infections (7/64, of which 3 HIV infections), immunodeficiencies (3/64), short bowel syndrome (3/64), congenital diarrhea (2/64), other/inconclusive diagnosis (8/64) (Fig. 1). Mean V/C ratio was 1.63. Forty-four, 15, and 5 showed Marsh 3a, 3b, and 3c lesion, respectively. The latter category included 2 patients with Crohn disease, 2 patients with immunodeficiencies, 1 with lymphohistiocytosis (Table 1).

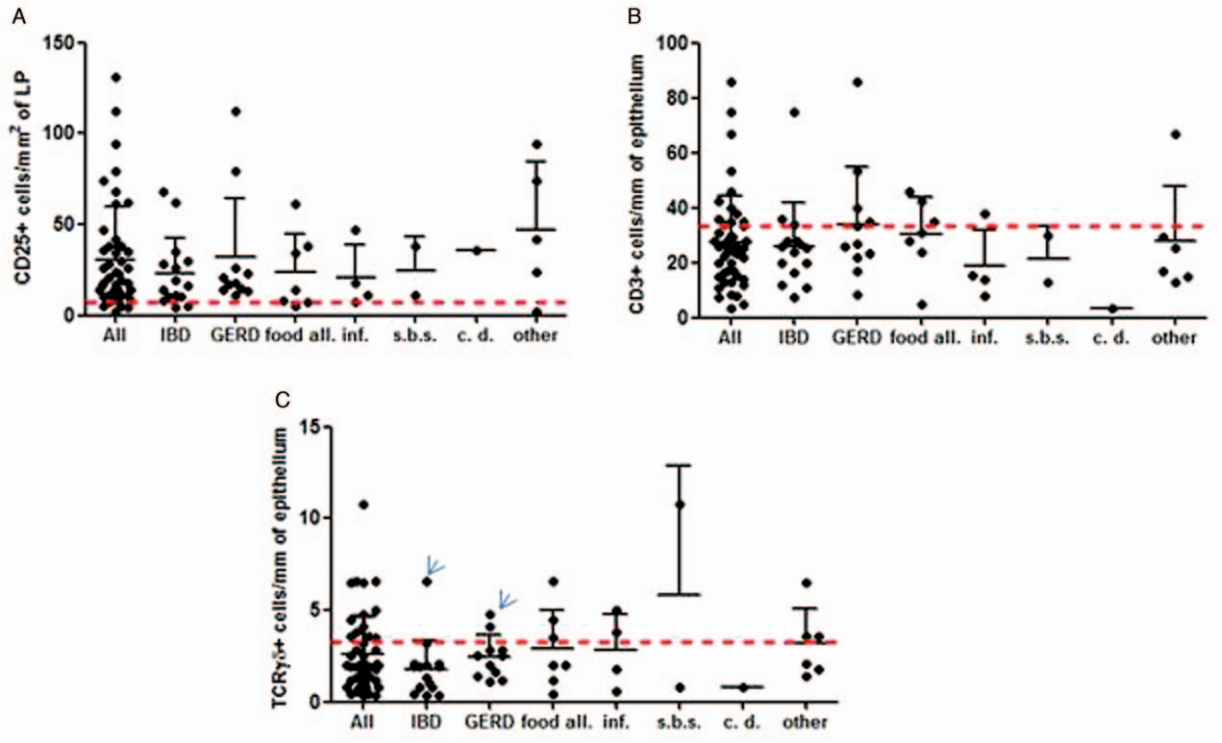

FIGURE 2. CD25+ mononuclear cells, CD3+ and TCRγδ+ intraepithelial lymphocytes density in duodenal mucosa of patients affected of nonceliac villous atrophy. The red lines represent cut-off values. IBD = inflammatory bowel disease; GERD = gastroesophageal reflux disease; SBS = short bowel syndrome; food all = food allergy; c.d. = congenital diarrhea; inf. = infections. The arrows indicate the 2 patients who had both positive staining for mucosal deposits of anti-TG2 antibodies and high TCRγδ+ cells.

Villous Atrophy Immunohistochemistry and Immunofluorescence Analysis

In 46 out 64 patients, immunohistochemistry on duodenal frozen sections has been performed. Lamina propria mononuclear cells expressing IL2 receptor (CD25 positive) were counted as marker of mucosal inflammation. In 41/46 (89%) patients, mononuclear CD25-positive cells were above the cut-off >4 cells/mm^2 (Fig. 2A). Intraepithelial lymphocytes expressing γδ T-cell receptor (TCR) were counted as high counts are indicative of gluten-dependent enteropathy. In 11/46 (24%), there was an excess (>34 cell/mm) of CD3+ intraepithelial lymphocytes (IELs) (Fig. 2B) and in 11/46 (24%) there was an excess (>3.4 cell/mm) of TCRγδ+ IELs (Fig. 2C). All subjects with elevated counts of TCRγδ+ IELs had in fact still low level of these cells (<3 times the cutoff of normality). Eighteen of 46 (39%) patients had CD3+ IELs and/or TCRγδ+ IELs above limits of normality. In 10/46 (22%) patients, a positive immunofluorescence indicated the presence of anti-TG2 mucosal antibodies: in 9 patients with a very weak staining and in 1 patient, with Crohn disease, with a weak staining and patchy distribution. In 2 cases, patients had concurrently a very weak positive staining for anti-TG2 mucosal antibodies and high TCRγδ+ IELs (Fig. 2C, arrows): 1 patient with GERD and 1 patient with Crohn disease.

DISCUSSION

Celiac disease remains the single most frequent cause of VA in the adult as well as in the pediatric population. Nonetheless, SNVA is not such a rare condition as it represents up to 5% of the diagnosis of VA in children. In most cases (70%), VA is mild (Marsh3a). One could argue that this could be a ''false'' VA, related to a not proper orientation of the duodenal biopsy. In fact, to correctly analyze biopsies, it is essential that the plane of the section is perpendicular to the luminal surface, as judged by the fact that the crypts of Lieberkuhn are cut longitudinally and not in cross section (13). To avoid this, together with qualitative histology (Marsh-Oberhuber classification), we also analyzed the quantitative ratio between villi and crypts, confirming the real presence of VA (mean V/C ratio 1.6). As in CD, most patients with a SNVA had an inflamed lamina propria, with an increase of CD25+ mononuclear cells (mainly lymphocytes and macrophages, expressing IL2 receptor and indicating an inflamed intestinal mucosa). This is not surprising as the mucosa remodeling leading to VA is a feature of many conditions sustained by T-cell activation, such as IBD and graft-versus-host reaction (14,15).

On the contrary, unlike CD, only about one quarter of patients had an increased number of IELs and of TCRγδ+ IELs. These cells typically increase in the epithelium of CD patients, and are thus considered as an immunohistochemical marker of CD. Moreover, all subjects with elevated counts of TCRγδ+ IELs had in fact relatively low level of these cells (<3 times the cutoff of normality).

A small proportion of patients (22%) present anti-TG2 mucosal antibodies, most of them with a weak intensity. Actually, we know that intestinal anti-TG2 antibody production does not show absolute specificity for CD. It has often been seen in association with inflamed mucosa. In a recent study, it has been shown that anti-TG2 mucosal antibodies can be present in up to 24% of seronegative patients with diagnosis other than CD (16).

In our cohort of patients, the single most frequent cause of SNVA is IBD. There could be, however, a selection bias as our center is the regional reference center. Surprisingly VA occurred similarly in Crohn and ulcerative colitis disease (11 vs 12 patients). In fact, even if only in Crohn disease, the upper gastrointestinal tract is generally macroscopically involved, duodenitis may develop in both Crohn and ulcerative colitis disease, even in the absence of upper gastrointestinal symptoms.

Other causes of SNVA are in the order food allergies (8/64), infections (7/64, of which 3 HIV infections), immune deficiency (3/64), short bowel syndrome (3/64), congenital diarrhea (2/64), others/inconclusive diagnosis (8/64). All these causes, because of their pathogenesis, are more frequent in pediatric then in adult patients, with the exception of the infections. On a more general note, it should be emphasized that most of those nonceliac biopsies show, a milder degree of remodeling in comparison to the more severe picture observed in CD.

The second most frequent clinical diagnosis associated with SNVA in our cohort is surprisingly represented by GERD. GERD should be more correctly considered as an association rather than the cause of VA. We putatively hypothesize that hyperacidity could be the link between GERD and VA, as the first part of the duodenum can be regarded as an extension of the gastric antrum and consequently it is exposed to acidic gastric secretions (17). As in GERD, the esophageal damage can be explained by an excess of gastric acid secretions, similarly we could speculate that this same acid causes a duodenal damage. This remains a hypothesis that should be investigated. In any case in our cohort of patients, clinical features, the mild degree of the lesions and the immunohistochemistry data (when available) did not make a trial with gluten-free diet necessary.

Our cohort of patients did not include other causes known to determine VA, such as autoimmune enteropathy, tropical sprue, drug-induced enteropathy, and seronegative celiac disease (SNCD). Some of these causes are extremely rare when taken singularly [for instance, autoimmune enteropathy prevalence is 1 : 100000 (18)] and some other are rare in pediatric age. For example, drug-induced enteropathy is typically related to the use of antihypertensive drug like angiotensin II receptor blocker (olmesartan), that are commonly used in adults but of course not in children (19).

Unlike the adult population, where seronegative celiac disease is the most frequent cause of SNVA (4–7), this condition seems to be virtually absent in pediatric age, as also stated in a recent article published by the Finnish group (20).

Of course, this is a retrospective study and, as such, has some limitations. For instance, all samples were not reviewed by the same pathologist and immunohistochemistry staining was not performed for all patients, which limits the ability to interpret these results. Like in all retrospective studies, causality links cannot be demonstrated and it remains to establish whether GERD is a cause of SNVA or a casual association. Moreover, the percentage of SNVA could partially be overestimated by the fact that, since the year 2012, diagnosis of CD can be made without intestinal biopsy in selected cases (presence of high level of anti-TG, symptoms and HLA DQ2, and/or DQ8 genetics) (21), which actually represented almost half of the cases in our practice. If we analyze separately data collected between 2010 and 2012, we observe that the percentage of SNVA decreases to 4% (19 cases of SNVA over 463 cases of VA).

In conclusion, SNVA remains an area of active clinical research and, given the heterogeneity of the clinical conditions associated to VA, it calls for studies aimed to validate biomarkers both for CD and non-CD conditions, which may improve diagnostic accuracy.

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