Seronegative Celiac Disease: Shedding Light on an Obscure Clinical Entity

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Abstract

Background: Although serological tests are useful for identifying celiac disease, it is well established that a minority of celiacs are seronegative.

Aim: To define the prevalence and features of seronegative compared to seropositive celiac disease, and to establish whether celiac disease is a common cause of seronegative villous atrophy.

Methods: Starting from 810 celiac disease diagnoses, seronegative patients were retrospectively characterized for clinical, histological and laboratory findings.

Results: Of the 810 patients, fourteen fulfilled the diagnostic criteria for seronegative celiac disease based on antibody negativity, villous atrophy, HLA-DQ2/-DQ8 positivity and clinical/histological improvement after gluten free diet. Compared to seropositive, seronegative celiac disease showed a significantly higher median age at diagnosis and a higher prevalence of classical phenotype (i.e., malabsorption), autoimmune disorders and severe villous atrophy. The most frequent diagnosis in the 31 cases with seronegative flat mucosa was celiac disease (45%), whereas other diagnoses were Giardiasis (20%), common variable immnodeficiency (16%) and autoimmune enteropathy (10%).

Conclusions: Although rare seronegative celiac disease can be regarded as the most frequent cause of seronegative villous atrophy being characterized by a high median age at diagnosis; a close association with malabsorption and flat mucosa; and a high prevalence of autoimmune disorders.

Key words: autoantibodies; autoimmune disorders; seronegative celiac disease; villous atrophy; olmesartan; common variable immunodeficiency; autoimmune enteropathy.

1. Introduction

Celiac disease (CD) is universally regarded as an immune-mediated enteropathy characterized by small intestinal villous atrophy occurring in genetically predisposed subjects [1-2]. In the last few decades, the identification of reliable serological biomarkers [3], i.e. anti-tissue transglutaminase (tTGA), endomysial (EmA) and deamidated gliadin peptide (DGP) antibodies, has progressively downgraded the prominent role of histology in CD diagnosis. In this respect, the ESPGHAN guidelines recommend skipping the duodenal biopsy in symptomatic children with high titer tTGA and positivity for genetic CD markers (i.e., HLA-DQ2 and/or –DQ8) [4]. Although CD-antibodies are detected in the vast majority of patients with CD (with an overall sensitivity ranging from 95% to 98%), a minority of CD patients may test negative for serology and in these cases the diagnosis is strictly dependent on the demonstration of villous atrophy at histopathology [5-6]. In these cases, HLA-DQ2 and/or -DQ8 positivity is a mandatory requirement to suspect the diagnosis of seronegative CD. Furthermore, both clinical and histological improvement should be proved after an adequate period of gluten-free diet (GFD). However, the finding of villous atrophy with negative CD serology is still a clinical challenge since severe small intestinal damage can be found in a variety of diseases other than CD, including parasitic infection (*Giardia lamblia*), autoimmune enteropathy, small intestine bacterial overgrowth (SIBO), common variable immunodeficiency (CVID), eosinophilic gastroenteritis, drug-induced enteropathy (mainly related to angiotensin II inhibitors), intestinal lymphoma, Crohn's disease, tropical sprue, human immunodeficiency virus (HIV) infection and Whipple disease [7-14]. Thus, prior to posing a firm diagnosis of seronegative CD, it is mandatory to rule out other causes of villous atrophy in order to avoid an unnecessary lifelong GFD.

Because of the paucity of data on seronegative CD, it is unclear whether this condition differs from CD with positive serology [15-17]. Also, the frequency of seronegative CD among the wide constellation of diseases associated with villous atrophy remains to be defined.

The aims of the present paper were threefold: 1) to define the prevalence of seronegative CD consecutively identified in a tertiary referral center; 2) to verify whether seronegative CD shows peculiar features, which sets it apart from the more commonly diagnosed seropositive CD; and 3) to establish the actual impact of seronegative CD amongst gluten unrelated disorders displaying villous atrophy.

2. Methods

2.1 Patients

During a 16-year-period (January 1998 - January 2014), 810 CD patients (630 females, F/M ratio 3.5:1, median age at diagnosis 36 years, range 18-78 years) were diagnosed at the tertiary referral CD Center of St. Orsola-Malpighi University Hospital (Bologna, Italy). The diagnostic process adopted to confirm CD included duodenal histopathology, serology and HLA typing (when necessary). Small intestinal biopsy results of two samples taken from the duodenal bulb and 2 from the second portion, were consistent with villous atrophy (mild, partial or total) in all the 810 patients [18]. Antibody testing was based on the identification of IgA tTGA and EmA; in cases with selective IgA deficiency, IgG tTGA were assayed. Serological tests have been always performed in the immunology laboratory of the St. Orsola-Malpighi University Hospital in Bologna. tTGA have been detected by ELISA using a home-made kit in the first three years (1998-2000), and a validated, standardized and reliable commercial kit (Eurospital, Trieste, Italy) in the remaining period (2001-2014). EmA detection was performed by indirect immunofluorescence on monkey oesophagus and human umbilical cord and the tests were always read by two blinded experts

(U.V. and R. De G.). A detailed HLA typing including HLA-DQ2.5 (DQA1*0501, DQB1*0201), HLA-DQ8 (DQA1*03, DQB1*0302), HLA-DQ 2.2 (DQA1*0201, DQB1*0202) and HLA-DQ7.5 (DQA1*05, DQB1*0301) has been performed in cases with discrepancy between histology and serology. Among the 810 CD patients, we retrospectively focused our attention on seronegative CD cases, comparing their clinical, histopathological and genetic features to those of seropositive CD. The clinical phenotype of CD was defined as classical (diarrhoea with malabsorption), non-classical (gastrointestinal symptoms other than diarrhea and extraintestinal manifestations) and subclinical (fully asymptomatic or with symptoms below the threshold of detection) [19]. Essential requirements to confirm the diagnosis of seronegative CD were both the positivity for HLA-DQ2 and/or -DQ8 and the regrowth of small intestinal villi detected in a second duodenal biopsy after at least 1-year of GFD. The frequency of CD amongst all patients with seronegative villous atrophy of any origin was also established. Since patients were not individually identified, a simplified International Review Board approval by the Ethics Committee of our Hospital was obtained.

2.2 Statistical analysis

Statistical analysis was performed by applying the Mann-Whitney U test to compare the age of patients at diagnosis in seronegative *vs.* seropositive CD. Moreover, the Pearson Chi-square test was used to compare the classical phenotype, the presence of total villous atrophy and the association with autoimmune disorders in seronegative *vs.* seropositive CD patients. The level of significance was set at $P<0.05$. Statistical evaluation was carried out using GraphPad Prism 3.0 (GraphPad Software Inc. San Diego, CA, USA).

3. Results

Of the 810 CD patients, 796 (98.3%) were seropositive (780 had IgA tTGA and/or EmA and 16 with selective IgA deficiency tested positive for IgG tTGA). On the whole, only 14 patients (1.7%) fulfilled the criteria for seronegative CD. The median age at diagnosis was 49 years (range 19-75 years) with a female gender predominance (12 women). HLA typing disclosed positivity for DQ2 in 12 cases (of which 5 showed homozygosity), whereas the remaining 2 were DQ8-positive. Total villous atrophy was observed in 8 out the 14 patients, whereas the remaining 6 had partial (n= 3) and mild (n= 3) villous atrophy. All the 14 patients with seronegative CD had a classical phenotype characterized by diarrhoea and severe malabsorption with a significant weight loss. Of these 14 patients, 4 (29%) had at least one relative with seropositive CD. Seronegative CD patients displayed a frequent association with autoimmune disorders, which were found in 6 (43%) of them and included Hashimoto thyroiditis (2 cases), primary biliary cirrhosis (1 case), autoimmune gastritis (1 case), gluten ataxia (1 case) and peripheral neuropathy (1 case) (Table 1). Concerning autoantibody profile, 7 patients had antinuclear antibodies (ANA), 1 had anti-mitochondrial, 1 anti-smooth muscle, 1 anti-gastric parietal cell and 2 anti-neuronal antibodies. On the whole, 10 (71%) out of the 14 seronegative CD patients showed at least one autoantibody positivity. Although CD serological markers, i.e EmA and tTGA, were negative, 4 out of the 14 patients tested positive for antibodies to native gliadin (AGA) of the IgG class (in one case associated with IgA positivity), nowadays no longer considered markers of CD (Table 1). The main differences between seropositive and seronegative CD are reported in Table 2. Compared to seropositive CD, seronegative CD showed a significantly higher median age at diagnosis (49 years *vs.* 36 years, $P< 0.005$) and a significantly more frequent classical phenotype (100% *vs.* 34%, $P< 0.001$). Both seronegative and seropositive CD were more frequent in female patients with a higher F/M ratio in the former *vs.* the latter group (F/M 6:1 *vs.* 3.5:1). Moreover, seronegative CD displayed total

villous atrophy and co-association with autoimmune disorders more frequently than seropositive CD, although these differences did not reach statistical significance.

Globally, 31 cases of seronegative villous atrophy were identified. The most frequent cause of flat mucosa in this subset of patients was seronegative CD, found in 14 cases (45%). In the remaining cases the final diagnosis was Giardiasis in 6 (20%), CVID in 5 (16%), autoimmune enteropathy in 3 (10%), SIBO in 1 (3%), olmesartan enteropathy in 1 (3%) and eosinophilic enteritis in 1 (3%) (Figure 1).

4. Discussion

The vast majority of CD patients display a wide array of serological biomarkers, namely tTGA, EmA and DGP, however CD can also occur in patients testing negative for CD serology [15-17, 20]. The existence of seronegative CD strengthens the importance of duodenal biopsy since the finding of villous atrophy in these patients represents the first step towards a correct diagnosis [21-22]. According to established guidelines [5, 23], patients with malabsorption and other related symptoms should always undergo a duodenal biopsy to rule out the occurrence of seronegative CD. The actual frequency of seronegative CD is still debated and discordant information is currently available on the clinical features of this CD subset [16]. Early studies found a high prevalence of seronegative CD associated with less severe intestinal damage and mild symptoms [6, 20]. A more recent paper, however, reported that seronegative CD was rare, mainly diagnosed in elderly people and associated with a severe histological and clinical involvement [15]. Compared to previous studies, the latter one used a more sensitive serological approach based on tTGA, which can partially explain the lower frequency of seronegative CD.

Moreover, the finding of villous atrophy in a patient with malabsorption lacking CD-antibodies is not necessarily indicative of seronegative CD, since many other disorders, mimicking the clinical

picture of CD, can cause villous atrophy [7-14]. The differential diagnosis between seronegative CD and other causes of villous atrophy is still a challenge with relevant implications in terms of outcome and treatment [15, 24].

Our retrospective study, based on a large cohort of CD, demonstrated that seronegative CD occurs in less than 2% of cases. On the whole, only 14 out of 810 consecutive CD patients fulfilled the criteria for seronegative CD. This small subgroup showed peculiar features setting them apart from the vast majority of seropositive CD patients. In fact, compared to seropositive CD, seronegative CD manifested the following features: 1) a significantly higher median age at diagnosis (49 *vs.* 36 years) with a female gender predominance; 2) a significantly higher prevalence of classical phenotype (diarrhoea and malabsorption); 3) a more frequent occurrence, although not reaching statistical significance, of autoimmune disorders along with a higher prevalence of autoantibodies; and, finally, 4) a higher (although not significant) frequency of total villous atrophy. Small intestinal biopsy was performed in all cases with intestinal symptoms (i.e. diarrhea, severe constipation, abdominal pain) and extraintestinal manifestations (i.e. anemia, unexplained osteoporosis) regardless the negativity of serological tests. Our data are in line with those reported by Degaetani et al. [15] showing that seronegative CD is a rare condition, diagnosed in elderly people with a severe malabsorption and intestinal damage. Regarding HLA typing, in our study a high number of seronegative CD patients showed positivity for HLA-DQ2 homozygosity, a genetic pattern closely associated with autoimmunity and increased risk of complications in CD [25-26]. Furthermore, a histological and clinical improvement after GFD was demonstrated in all cases with seronegative CD. Interestingly, about one third of patients with seronegative CD had at least one relative with seropositive CD. Although non-specific, the finding of AGA of the IgG class helped to guide diagnosis in a small subset (n= 4) of seronegative CD. Therefore, one might speculate that AGA positivity in seronegative villous atrophy can suggest an underlying gluten

related etiology. Also, AGA are regarded as possible markers of non-celiac gluten sensitivity, a new gluten-related syndrome showing a normal or mildly inflamed small intestinal mucosa [27].

Since our study started in 1998, we did not include in our protocol a number of immunological tests identified in more recent years as possible markers of CD, such as serum DGP [28], serum tTGA with open conformation [29], EmA detected in small bowel mucosa organ system [30] and IgA intestinal deposits of tTG [31]. All these tests are not routinely use for clinical purposes, with the exception of DGP, which are nowadays recommended in the first infancy and in IgA deficiency [3].

After an initial good clinical response, two patients with seronegative CD (case 3 and 5 in Table 1) worsened regardless of a strict adherence to GFD, developing in one case refractory CD and in the other one a neurological complication. Based on the poor outcome of these two cases it is tempting to hypothesize that seronegative CD might be a condition susceptible to complications.

Our results demonstrated that seronegative CD is the most frequent diagnosis among the wide spectrum of disorders causing villous atrophy unrelated to CD serology. This finding confirms and expands previously published data [9, 15]. In our experience, seronegative CD accounted for 14 (45%) out of the 31 patients presenting with villous atrophy without celiac serology. The second largest group included Giardiasis (20%), followed by CVID (16%), and autoimmune enteropathy (10%). Among the disorders included in the differential diagnosis with CD, CVID deserves a special attention. Indeed, a minority of CVID patients with HLA-DQ2 and/or –DQ8 positivity can have a concurrent CD. This peculiar association can be proven by a positive clinical response to GFD. In contrast to previous data [15], olmesartan-induced enteropathy was rarely observed in our series. Other disorders, which can potentially determine villous atrophy, e.g. Whipple disease, tropical sprue, Crohn's disease, and HIV infection, were not observed in our series.

The detection of seronegative villous atrophy requires a thorough work-up and a possible clinical / diagnostic algorithm is illustrated in Figure 2. HLA-DQ2 and -DQ8 should be determined since the negativity of this test rules out seronegative CD [25]. In contrast, the finding of DQ2 and/or -DQ8 positivity guides towards CD; however, before starting a trial with GFD, other etiologies of villous atrophy should be excluded regardless the genetic pattern. Enterocyte autoantibodies, immunoglobulins (IgG, IgA and IgM), and HIV should be investigated to rule out autoimmune enteropathy, CVID and HIV infection, respectively [10-11, 32]. Lactulose breath test for SIBO and detection of *Giardia lamblia* in the stools and biopsy specimens should be performed [8]. The pathologist should consider all causes leading to villous atrophy [7]. Moreover, an accurate drug review in the patient's clinical history is mandatory to identify any possible drugs involved in villous damage, i.e. olmesartan and non-steroidal anti-inflammatory drugs [8, 12]. Once all these potential causes of villous atrophy have been excluded, patients with a genetic pattern consistent with CD should start GFD. The clinical and histological response to gluten withdrawal is confirmatory of seronegative gluten enteropathy.

In conclusion, seronegative CD represents a very small subgroup inside the large population of seropositive CD. However, our data clearly indicate that it is the most frequent cause of seronegative villous atrophy. This clinical subgroup of gluten-related enteropathy has been (and still is) commonly overlooked, likely because of the surmounting relevance of serology for CD diagnosis. Furthermore, the results of the present study support the role of duodenal biopsy as the cornerstone for identifying seronegative CD. All patients with severe malabsorption should undergo a duodenal biopsy even if serological tests for CD are negative. A proportion of them can have seronegative CD, which requires a strict GFD. Clearly, the diagnosis of seronegative CD needs to be carefully confirmed by excluding other gluten-independent causes of villous atrophy in order to avoid an unnecessary, lifelong GFD.

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Figure legends

Figure 1.

The figure illustrates various etiologies of seronegative villous atrophy identified at the tertiary referral Celiac Disease Center of the St. Orsola-Malpighi University Hospital (Bologna, Italy). Among the various conditions characterized by villous atrophy of the duodenal mucosa without celiac markers, it is noteworthy that seronegative celiac disease subset represents the predominant one. Abbreviations: CD, celiac disease; CVID, common variable immune deficiency; SIBO, small intestine bacterial overgrowth.

Figure 2.

Proposed algorithm for the management of patients with seronegative villous atrophy. HLA-DQ2 and -DQ8 typing should be determined: if negative, this test rules out celiac disease (CD); DQ2 and/or -DQ8 positivity in a patient with villous atrophy indicate(s) a possible diagnosis of CD. Before starting GFD, other etiologies of villous atrophy should be excluded by testing enterocyte autoantibodies, immunoglobulins (IgG, IgA and IgM), HIV antibodies, *Giardia lamblia* in the stools and biopsy specimens, as well as lactulose breath test for SIBO. An accurate drug review is mandatory to identify any drugs possibly involved in villous damage, e.g. olmesartan. Biopsy histopathology should identify enteropathy-associated T cell lymphoma (EATL), collagenous sprue, eosinophilic enteropathy and Whipple disease. The exclusion of other conditions causing seronegative villous atrophy guides clinicians to a diagnosis of CD, which will be confirmed by the clinical and histological improvement after gluten free diet (GFD).

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Conflict of interest

The authors declare no conflict of interest.

All authors approved the final version of the manuscript. Also, the present work has not been submitted elsewhere.

Conflict of interest

The authors declare no conflict of interest.

Table 1. Clinical features, histology, genetics and associated disorders in patients with seronegative celiac disease.

Pts	Gender	Age at diagnosis	Clinical phenotype	HLA	Duodenal biopsy		tTGA EmA	AGA	Associated disorders	Other autoantibodies	CD familiarity
					Untreated	After GFD					
#1	Male	58	Classical	DQ2+ homozygosis	3c	1	Neg.	IgG	Autoimmune thyroiditis	ANA homogeneous 1:160	none
#2	Female	34	Classical	DQ2+	3a	1	Neg.	IgG	PBC	AMA M2	2 sons
#3	Female	37	Classical	DQ2+	3c	0	Neg.	Neg.	None	none	none
#4	Female	75	Classical	DQ2+ homozygosis	3c	1	Neg.	Neg.	None	none	2 nephews
#5	Female	45	Classical	DQ2+	3a	1	Neg.	Neg.	None	none	none
#6	Female	55	Classical	DQ2+ homozygosis	3c	1	Neg.	IgG	Gluten ataxia	ANA speckled 1:160, CNS	2 sisters
#7	Male	61	Classical	DQ8+	3b	1	Neg.	Neg.	Peripheral neuropathy	CNSA, ENSA	none
#8	Female	48	Classical	DQ2+	3c	1	Neg.	Neg.	None	none	none
#9	Female	49	Classical	DQ2+ homozygosis	3a	1	Neg.	IgG and IgA	Autoimmune gastritis	HPCA ANA homogenous 1:640	none
#10	Female	30	Classical	DQ2+	3b	0	Neg.	Neg.	None	ASMA	none
#11	Female	63	Classical	DQ2+	3c	1	Neg.	Neg.	None	ANA speckled 1:160	none
#12	Female	46	Classical	DQ2+ homozygosis	3c	1	Neg.	Neg.	None	ANA speckled 1:80	1 son
#13	Female	19	Classical	DQ2+	3c	1	Neg.	Neg.	Neg	ANA speclked 1:160	none
#14	Female	40	Classical	DQ8+	3b	1	Neg.	Neg.	Autoimmune thyroiditis	ANA speckled 1:320	none

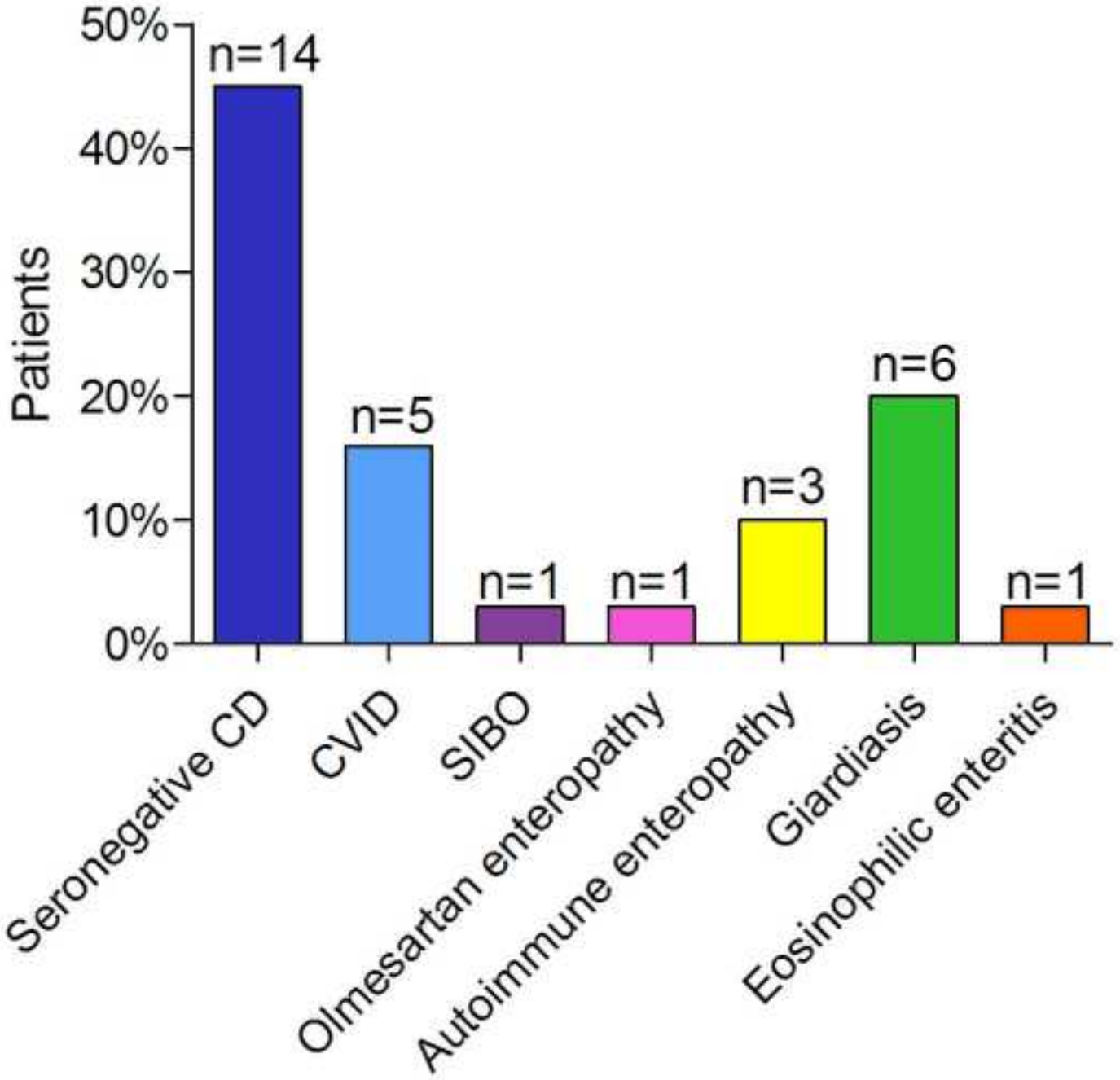
Note: tTGA: tissue transglutaminase antibodies, EmA: endomysial antibodies, AGA: antibodies to native gliadin; GFD, gluten-free diet; PBC, primary biliary cirrhosis; ANA: antinuclear antibodies on HEp-2 cells; CNSA: central nervous system autontibodies; ENSA: enteric nervous system autontibodies; HPCA: human parietal cell autoantibodies; ASMA: anti smooth muscle antibodies; duodenal biopsy scored according to Marsh-Oberhüber classification: "3a", mild; "3b", partial; "3c", total villous atrophy; "1" indicates increased intraepithelial lymphocytes (>25/100).

Table 2. Comparison between seronegative and seropositive coeliac disease (CD).

	(A) Seronegative CD (14 cases)	(B) Seropositive CD (796 cases)
Gender (F/M ratio)	6:1	3.5:1
Median age at onset	49 years (range 19-75)	36 years (range 18-78)
Classical phenotype (diarrhea, malabsorption, weight loss)	100%	34%
Total villous atrophy	57%	36%
Autoimmune disorders	43%	30%

Median Age at onset in A *vs*. B: $P < 0.005$; Classical phenotype in A vs B: $P < 0.001$; Total villous atrophy in A *vs*. B: *P*= ns; Autoimmune disorders in A *vs*. B: *P*= ns.

Figure 1


50%
40%
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Patients
n=14
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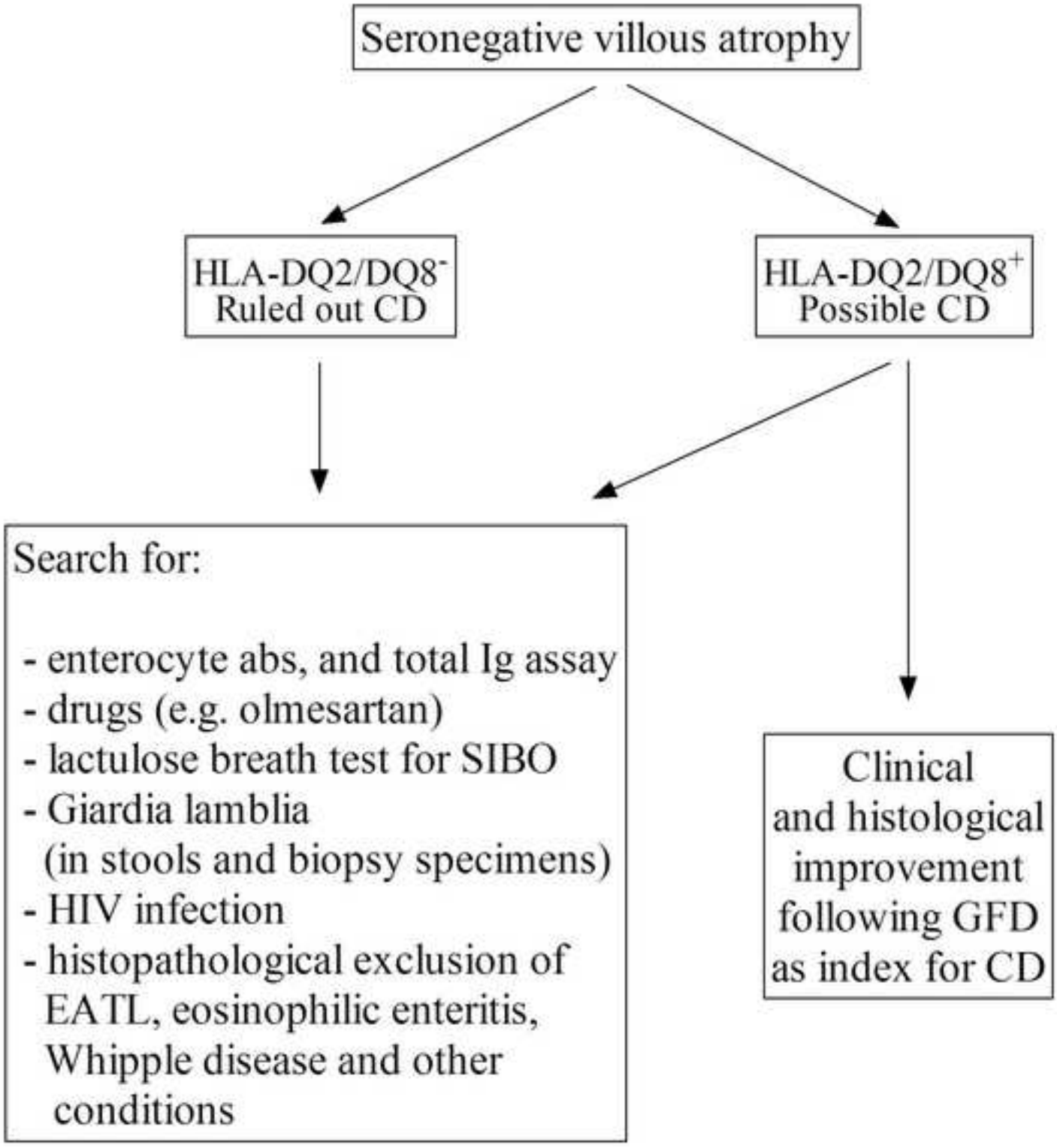
Seronegative CD
CVID
SIBO
Olmesartan enteropathy
Autoimmune enteropathy
Giardiasis
Eosinophilic enteritis

Figure 2


Seronegative villous atrophy
HLA-DQ2/DQ8- Ruled out CD
HLA-DQ2/DQ8+ Possible CD
Search for:
- enterocyte abs, and total Ig assay
- drugs (e.g. olmesartan)
- lactulose breath test for SIBO
- Giardia lamblia (in stools and biopsy specimens)
- HIV infection
- histopathological exclusion of EATL, eosinophilic enteritis, Whipple disease and other conditions
Clinical and histological improvement following GFD as index for CD