

## CLINICAL STUDY

## Markers of potential coeliac disease in patients with Hashimoto's thyroiditis

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### Abstract

**Objective:** Coeliac disease (CD) is associated with autoimmune thyroid disease. Gluten sensitivity represents a spectrum, with at one end cases with severe gluten-dependent enteropathy, and at the other subjects with minor signs of deranged mucosal immune response. The aim of this paper was to look for signs of minor small bowel injury and immunohistochemical markers of gluten sensitivity in a group of patients with Hashimoto's disease.

**Subjects and methods:** Fourteen patients with Hashimoto's thyroiditis without serological evidence of CD underwent immunohistochemical analysis of jejunal biopsies.

**Results:** In 6/14 cases (43%) an increased density of  $\gamma\delta$  T cell receptor bearing intra-epithelial lymphocytes was found. In 6/14 (43%) signs of mucosal T cell activation (presence of interleukin 2 (IL2) receptor (CD25) on lamina propria T cells and/or expression of human lymphocyte antigen (HLA)-DR molecules on crypt epithelial cells) were noted. In 4 out of 6 such cases, HLA haplotypes were described in association with CD.

**Conclusion:** A significant proportion of patients with Hashimoto's thyroiditis present signs of 'potential' CD and of activated mucosal T cell immunity. The gluten dependence of such findings remains to be ascertained.

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### Introduction

Coeliac disease (CD), also named gluten-sensitive enteropathy, is a disorder in which prolamines from wheat, rye, barley and possibly oats, cause small bowel mucosal damage; the enteropathy is sustained by an abnormal immune response to gliadin and related prolamines, in genetically susceptible individuals (1). The diagnosis relies on the demonstration, on a gluten-containing diet, of typical small bowel pathology (villous atrophy, crypt hyperplasia, increased density of inflammatory cells both in epithelium and lamina propria); the presence of serum anti-endomysium and anti-tissue transglutaminase antibodies, and of certain human lymphocyte antigen (HLA) haplotypes known to be associated with CD, lend support to the diagnosis (2). The prevalence of CD, as suggested by studies based on serological screening, is as high as 1:100 (3) with a wide range of clinical presentations. Furthermore, CD is associated with other conditions, primarily autoimmune diseases (4). Endocrine autoimmune diseases (autoimmune thyroid disorders (ATD), insulin-dependent diabetes mellitus (IDDM)

and Addison's disease) are the conditions most frequently associated with CD (5).

Over the last decade it has become clear that the term CD should not be limited to patients with severe enteropathy, but it should be applied also to patients with mild, sometimes patchy, enteropathy. At the end of the spectrum of 'gluten sensitivity' there are subjects with normal small bowel morphology, but with features of deranged mucosal immune response (6–8); among the latter there are immunohistochemical signs, such as an excess of  $\gamma\delta$  T cell receptors (TCR) bearing intra-epithelial lymphocytes, that have been shown in some cases to precede the development of villous atrophy (9).

In a previous study (10) we used an endomysial antibody to screen 150 patients with autoimmune thyroid disease; five showed a positive serology and were diagnosed as coeliacs on the basis of the jejunal biopsy. The aim of this paper was to look for signs of minor small bowel injury and immunohistochemical markers of gluten sensitivity in a group of ATD patients, in particular patients with Hashimoto's thyroiditis, without serological markers of CD.

## Subjects and methods

### Patients

Fourteen adult patients with Hashimoto's disease, 2 males and 12 females, with a median age of 48 years (range 17–77 years), were enrolled in this study, after informed consent, in accordance with the Helsinki Declaration. The diagnosis of Hashimoto's disease was made on the basis of ultrasonography that revealed a diffuse reduction in thyroid echogenicity, high thyrotrophin (TSH) and low thyroid hormone levels, the presence of high levels of antithyroid antibodies, such as thyroid peroxidase (TPO) and/or anti-thyroglobulin (AbHTG) antibodies, and lymphocytic thyroid infiltration established by fine needle aspiration. All patients were on substitutive therapy with L-thyroxine for the primary hypothyroidism, in different doses (range 75–125 µg/day) for at least one year.

One of the patients had Sjogren's syndrome associated with Hashimoto's thyroiditis. None had a history of steatorrhoea, but five had some mild abdominal complaints.

### Jejunal biopsy and immunohistochemical analysis

All subjects underwent upper gastrointestinal endoscopy and small bowel biopsy. Four forceps biopsy specimens were obtained from the distal part of the duodenum, two for morphological routine examination and two for immunohistochemical staining. For the histological and morphometrical techniques, the biopsy specimens were stained with haematoxylin and eosin.

For the immunohistochemical study, biopsy specimens were immediately embedded in optimal cutting temperature compound (OCT) (BioOptica, Milan, Italy) and stored at  $-80^{\circ}\text{C}$ . Cryostat sections were cut at  $4\ \mu\text{m}$  and fixed in acetone for 10 min. After a 20-min preincubation with normal rabbit serum (Dako, Copenhagen, Denmark), sections were covered for 1 h with anti-CD3 (1:200; Dako), anti-CD25 (1:20; Dako), anti-TCR $\gamma\delta$  (1:80; Thema, Bologna, Italy), anti-HLA-DR (1:10; Dako) monoclonal antibodies, followed by rabbit anti-mouse immunoglobulins for 30 min. Monoclonal antibodies were diluted in Tris pH 7.4; all incubations were performed at room temperature in a humid chamber. As a negative control, primary monoclonal antibody was replaced with mouse IgG2a/IgG1 (Dako). After washing with Tris pH 7.4, the sections were layered with monoclonal mouse peroxidase anti-peroxidase (PAP) (Dako) or monoclonal mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) (Dako) for 30 min. 3-Amino-9-ethylcarbazole (AEC; Sigma, St Louis, USA) or new fuchsin were used as peroxidase and phosphatase alkaline substrates respectively.

Finally, sections were counterstained with Mayer's haematoxylin and mounted with Aquamount (BDH, Milan, Italy).

### Morphometric analysis

The density of cells expressing CD3 and TCR $\gamma\delta$  in the intra-epithelial compartment was determined by counting the number of stained cells per mm epithelium; the number of CD25 of lamina propria mononuclear cells was evaluated within a total area of  $1\ \text{mm}^2$  of lamina propria, using a microscope with a calibrated ocular aligned parallel to the muscularis mucosae. Staining of epithelial cells by anti-HLA-DR was evaluated in terms of staining intensity, and graded on an arbitrary scale of weak staining (+) = 1, moderate staining (++) = 2, strong staining (+++) = 3. The counts were independently analysed in a blinded manner by 2 observers.

### Serology

IgA- and IgG-class anti-gliadin antibodies (AGA) were tested by enzyme-linked immunosorbent assay (ELISA). IgA-class anti-endomysium antibodies were revealed by an indirect immunofluorescence method, using unfixed cryostat sections of human umbilical cord as substrate (11). Anti-tissue-transglutaminase antibodies were measured by ELISA, as described elsewhere (12). Finally, TPO and AbHTG antibodies were measured using commercial kits, radioimmunoassay (RIA) and immunoradiometric assay (IRMA) methods respectively (Radim, Pomezia-Roma, Italy).

### HLA typing

Molecular HLA typing has been performed as previously described (13). Briefly, DNA extraction was carried out according to a rapid extraction method. Amplification of the polymorphic second exon of DRB1, DQA1, DQB1 genes and dot blot analysis of amplified DNA with sequence specific oligonucleotides probes (SSO) was carried out.

### Statistics

The immunohistochemical data obtained in Hashimoto's thyroiditis patients were compared with those obtained in a group of 50 subjects whose final diagnoses were short stature, gastroesophageal reflux, iron deficiency anaemia, irritable bowel. In this group, for each parameter centiles were calculated using the SSPS software (Statistical Package for Social Science, release 8.0, Chicago, USA). Values were considered abnormal when exceeding the 90th centile of the control group.

**Table 1** Immunohistochemical analysis of small intestinal biopsies from patients with Hashimoto's thyroiditis. Characters in bold are values exceeding the 90<sup>th</sup> centile of the control group.

Patient	CD3 (n.v. < 32/mm epithelium)	TCR $\gamma\delta$ (n.v. < 3.2/mm epithelium)	CD25 (n.v. < 4.0/mm <sup>2</sup> lamina propria)	Crypt HLA-DR (n.v. neg.)
1	23	<b>7.4</b>	3	+
2	17	1.6	<b>6</b>	+
3	17	1.3	3	Neg.
4	8	2.2	2	Neg.
5	<b>44</b>	0.4	0	+
6	7	0.1	0	Neg.
7	22	0.2	1	+
8	<b>90</b>	<b>7.1</b>	<b>5</b>	+
9	<b>65</b>	<b>5.2</b>	2	+
10	14	0.1	3	Neg.
11	<b>79</b>	<b>5.1</b>	2	Neg.
12	<b>45</b>	<b>7.7</b>	1	Neg.
13	<b>34</b>	<b>6.0</b>	1	Neg.
14	<b>44</b>	0.2	0	Neg.

n.v., normal value; Neg., negative.

## Results

### Jejunal biopsy and immunohistochemical analysis

None of the subjects had morphological alteration of the jejunal mucosal architecture; in three cases there was a significant intra-epithelial infiltration.

The immunohistochemical analysis revealed increased density of intra-epithelial CD3+ cells in 7/14 patients with Hashimoto's thyroiditis. Six showed increased density of intra-epithelial  $\gamma\delta$ + cells, and five showed increased density of both CD3+ and  $\gamma\delta$ + cells (Table 1).

When markers of mucosal T cell activation were considered, 6/14 patients showed increased expression of HLA-DR molecules on crypt epithelial cells, but only in 2 cases was a slight increase in the density of lamina propria cells expressing interleukin 2 (IL2) receptor (CD25+ cells) observed (Table 1).

**Table 2** HLA haplotypes in 10 of the patients with Hashimoto's thyroiditis. Characters in bold and underlined are haplotypes known to be associated with coeliac disease.

Patient	DRB1*	DQA1*	DQB1*	DRB1*	DQA1*	DQB1*
5	03	<b>0501</b>	<b>02</b>	04	0301	03
6	07	0201	<b>02</b>	11	<b>0501</b>	03
7	07	0201	02	13	0103	03
8	<b>04</b>	0301	<b>03</b>	13(11)	0103	04
9	<b>04</b>	0301	<b>03</b>	04	0301	06
10	<b>04</b>	0301	<b>03</b>	13	0103	06
11	03	<b>0501</b>	<b>02</b>	14	0101	05
12	<b>04</b>	0301	<b>03</b>	11(13)	0501	03
13	07	0201	02	07	0201	02
14	11	0501	03	14	<b>0101</b>	<b>05</b>

### Serology

IgA and IgG antibodies, antigliadins, were within the normal range in all cases. None of these patients had endomysial antibodies in their serum. Also IgA anti-tissue transglutaminase levels were in the normal range; in 2 cases borderline titres were noted.

### HLA typing

Three patients (numbers 5, 6 and 11) presented the HLA DQ heterodimer most frequently associated with coeliac disease (A1\*0501, B1\*0201) (14), four (numbers 8, 9, 10 and 12) were DRB1\*04 positive, the latter being the haplotype more commonly present in CD patients negative for the A1\*0501, B1\*0201 heterodimer (14); finally, one (number 14) subject, negative for these previous haplotypes, was positive for an heterodimer found in a minority of CD patients (A1\*0101, B1\*0501) (13). The prevalence of DQA1\*0501/DQB1\*0201 in the general population of our region (15) is 27% compared with a prevalence of 30% in our small group of Hashimoto's thyroiditis patients. Patients carrying the DRB1\*04, the other HLA allele specificity also associated with coeliac disease, make up 17% of our general population, but significantly more were represented in the present group of Hashimoto's thyroiditis patients (40%).

Of the six subjects with increased density of  $\gamma\delta$ + cells, four had HLA haplotypes compatible with coeliac disease (Table 2).

### Clinical features

No differences were noted between patients with or without immunohistochemical mucosal changes suggestive of potential coeliac disease, when nutritional parameters, anthropometric indexes, age at presentation

of thyroiditis, number of autoimmune diseases and clinical control of the disease were considered.

## Discussion

Several reports, including our own (10), suggest a significant association between coeliac disease and autoimmune endocrinological disease (5, 16–18). It is still unclear if such an association, as well as the association with other autoimmune diseases, is fully explained by a shared immunogenetic make-up, or if a causative role of gluten may play a role, as suggested by the significant correlation observed in coeliac patients between the increased occurrence of autoimmune diseases and length of exposure to gluten (19).

The current diagnostic criteria for coeliac disease are based on the finding of small bowel mucosal villous atrophy with crypt hyperplasia, and on the gluten dependence of such a lesion (2). Evidence suggests that small bowel mucosal damage in coeliac disease develops gradually from mucosal inflammation to crypt hyperplasia, and finally to partial and subtotal villous atrophy (20). It is now accepted that coeliac disease may be characterized by more subtle histological changes; in some subjects only epithelial infiltration may be present (21); sometimes the lesion is patchy; in most cases endomysial antibodies are present, but not in all. More often there are immunohistochemical markers peculiar to coeliac disease, such as increased density of  $\gamma\delta$ + intra-epithelial T cells. From a genetic point of view such signs have been reported to be correlated to the presence of HLA alleles usually associated with coeliac disease (22), but there are reports of non-familial cases of mild gluten-dependent enteropathy with HLA alleles different from the classical ones (23).

The term 'potential' coeliac disease has been proposed to describe patients with normal or low-grade pathology and other subtle immunological abnormalities (e.g. coeliac-like mucosal immunoglobulin pattern, increased density of  $\gamma\delta$ + intra-epithelial T cells, positive response to rectal gluten challenge), meaning that they are at significant risk of developing coeliac disease later (6). Examples of the existence of such subjects come from the investigation of 'at-risk' groups, primarily first-degree relatives of coeliac patients. Among the latter there are subjects with architecturally normal jejunal mucosa, but increased density of  $\gamma\delta$ + intra-epithelial T cells (22) and others with a positive response to rectal gluten challenge (24). Similarly, in patients with IDDM there are subjects with signs of mucosal inflammation (25), although the gluten dependence of such abnormalities has never been assessed, and other patients with a positive response to rectal gluten challenge (26). More recently, a study of small bowel biopsies in Sjogren's syndrome has confirmed the strong association of this condition

with coeliac disease, and also the presence in a significant number of patients of enhanced mucosal HLA-DR expression (27).

The results obtained in our study confirm the presence of immunological abnormalities in the small bowel mucosa of another group of patients suffering from a condition associated with coeliac disease. Fifty percent of subjects showed an infiltrative pattern characterised by increased density of CD3+ intra-epithelial cells. More interesting for the correlation with coeliac disease is the finding that in 46% of subjects there is an increased density of  $\gamma\delta$ + intra-epithelial T cells. Similar observations have recently been reported in some patients with multiple autoimmune endocrinological disorders (5). The biological meaning of the increased density of  $\gamma\delta$ + intra-epithelial T cells is far from clear, but such findings have been described in subjects who have later developed a frank picture of severe enteropathy (9).

The immunohistochemical analysis conducted in subjects with Hashimoto's thyroiditis has also evaluated the presence of signs suggesting mucosal T cell activation. In 46% of subjects an increased expression of HLA-DR molecules on the crypt epithelium has been found, accompanied in two of these cases by the increased expression of IL2 receptor on lamina propria mononuclear cells; these findings suggest increased local production of proinflammatory cytokines, such as gamma interferon. The extent of such changes was less than that reported in insulin-dependent diabetes (25) or in Sjogren's syndrome (27). It is unclear if it is a phenomenon due to the autoimmune process, or if it is related to the presence of gluten in the diet. As there is little evidence that patients with minor small intestinal histological changes will benefit from a gluten-free diet, these subjects were not prescribed a gluten-free diet; nonetheless, they remain under surveillance.

In conclusion, our data show subtle mucosal changes in a group of subjects with Hashimoto's thyroiditis. They cannot be defined as coeliacs due to the absence of significant mucosal abnormalities and of typical serology (positive endomysial and anti-tissue transglutaminase antibodies). The increased density of  $\gamma\delta$ + intra-epithelial T cells in subjects with HLA haplotypes usually associated with gluten-dependent enteropathy suggest a condition of 'potential' coeliac disease. There is a strong consensus that patients with autoimmune thyroiditis should always be actively investigated for the possible coexistence of coeliac disease; our data suggest also that those serologically negative may carry a risk of developing the disease in the future. These observations, and the possibility, well documented in subjects with IDDM, of sero-conversion with time (28), impose the necessity of careful continuous serological surveillance in these patients.

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