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ORIGINAL ARTICLE

The contribution of intraepithelial inflammatory cells to the histological diagnosis of microscopic esophagitis

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Abstract
Background Diagnosis of gastro-esophageal reflux disease (GERD) is mainly based on symptom evaluation, possibly coupled with various invasive investigations (endoscopy, pH-metry, and impedance). New input has recently come from histology: in this context, the diagnostic role of inflammatory cells other than eosinophils and neutrophils is still debated. Aim of the study is to evaluate the diagnostic relevance of intraepithelial lymphocytes, mast cells, and Langerhans cells in GERD-associated microscopic esophagitis (GAME).
Methods Twenty healthy volunteers and 119 patients with GERD symptoms were prospectively recruited and subdivided, on the basis of endoscopy and pH-metry, in erosive (ERD, 48) and non-erosive disease (NERD, 71). Biopsy samples at 2 cm above the Z-line and at Z-line were evaluated for GERD-associated histological lesions (basal cell hyperplasia, papillae elongation, intercellular space dilatation, intraepithelial eosinophils, and neutrophils). Immunohistochemistry for T lymphocytes (CD3, CD4, CD8), B lymphocytes (CD20), NK cells (CD56), macrophages (CD68), mast cells (c-Kit), and Langerhans cells (S100) was performed.
Results Among inflammatory cells, only intraepithelial T lymphocytes (ITLs) showed statistical correlation with the other histological lesions both in ERD and NERD. ITLs distinguished GERD patients from controls with good sensitivity and specificity (85.5 and 85 % at 2 cm above Z-line; 89.5 and 75 % at Z-line) when a cut-off of 20 cells was applied. An analysis of the T subpopulations found a CD4+/CD8+ ratio close to 1:1; B cells, mast cells, Langerhans cells, NK cells, and macrophages showed a limited role in GERD.
Conclusions ITL evaluation represents an additional useful parameter in the histological evaluation of GAME.

Keywords Microscopic esophagitis · Histology · Inflammatory cells

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Introduction

Gastro-esophageal reflux disease (GERD) is a chronic disorder resulting from prolonged exposure of squamous esophageal epithelium to gastric or gastro-duodenal reflux. Typical symptoms are present in less than 50 % of patients with GERD [1]; clinical manifestations can vary greatly, ranging from symptoms without visible endoscopic lesions (non-erosive reflux disease—NERD) to erosive esophagitis (erosive reflux disease—ERD) and complications such as Barrett's esophagus and adenocarcinoma [2]. The prevalence of GERD in western countries is high, with reported rates of reflux symptoms ranging from 20 to 30 %, while in Asia, lower values (5–10 %) are reported [3–5]. This high prevalence, coupled with a negative impact on quality of life and significant health costs, makes GERD a major healthcare issue [6]. In this context an accurate diagnosis represents a challenge. Diagnosis is mainly based on

symptoms and clinical questionnaires are commonly used; when proton pump inhibitor therapy fails, several invasive methods (i.e., endoscopy, pH-metry, and impedance) can contribute to diagnosis with varying sensitivity and specificity. Recently, new input has come from histology as well. After the description of dilated intercellular spaces as a new elementary lesion capable of distinguishing patients with GERD from control subjects [7–9], several studies, aimed at reassessing the diagnostic value of histology in GERD, have been published [10–13].

The histological diagnosis of GERD-associated microscopic esophagitis (GAME) is based on the presence of several lesions, i.e., basal cell hyperplasia (BH), papillae elongation (PE), dilatation of intercellular spaces (DIS), intraepithelial eosinophils (IE) and neutrophils, and erosions/healed erosions [10, 12, 13]. None of these elementary lesions is "per se" diagnostic of GAME and diagnosis derives from the combined assessment of such lesions; the use of a score obtained by summing the severity of assessable lesions has also been suggested [10]. In this context, the diagnostic role of inflammatory cells other than eosinophils and neutrophils is still debated. A possible role for lymphocytes and plasma cells in the diagnosis of GAME was originally postulated by different authors [14–16] who described the presence of inflammation in the lamina propria as a marker of GAME in the absence of epithelial erosions or ulcers. Only with the seminal work by Ismail-Beigi [17], demonstrating the diagnostic relevance of architectural lesions (such as BH and PE) in subjects with reflux symptoms, was the importance of inflammatory cells in the diagnosis of GAME reconsidered. Many authors investigated the role of intraepithelial lymphocytes [17–22], but only a few have tried to investigate them in GERD by using immunohistochemistry [23]. Little information is available on the diagnostic relevance of other inflammatory cell types, i.e., mast cells and Langerhans cells; their role in esophageal damage has been hypothesized by some investigators both in animal models [24, 25] and in humans [23, 26, 27].

This study is aimed at the evaluation of the diagnostic relevance of inflammatory cells, and in particular intraepithelial lymphocytes, mast cells and Langerhans cells, in GAME by using immunohistochemistry for their identification and by comparing their presence in ERD and NERD patients with an appropriate control group of healthy volunteers.

Materials and methods

Study group

The study was carried out on a series of 119 consecutive patients (68 M/51 F, mean age 52 years, range 22–76) with typical or atypical symptoms of GERD, prospectively recruited in our open-access endoscopy service. The clinical, instrumental, and histological methodological approach has been detailed previously [11, 28]. Briefly, all patients agreed to undergo both endoscopy and 24-h esophageal pH monitoring. Typical symptoms included heartburn and/or regurgitation and were present in 99 patients; atypical symptoms (i.e., chest pain, asthma etc.) were found in the remaining 20 patients: the enrolment of these last patients was dependent on the presence of a positive PPI test. Frequency, intensity, and impact of symptoms on quality of life were registered by using a validated questionnaire [29]. Antisecretory or prokinetic drugs were stopped at least 30 and 15 days, respectively, before endoscopy. On the basis of endoscopy, patients were divided in ERD (n°48) and NERD (n°71); NERD patients were further subdivided on the basis of pH testing in NERD patients with pathologic pH test (NERD pH+, n°59) and NERD patients with normal pH values (NERD pH−, n°12). Twenty subjects (12 M/8 F, mean age 51 years, range 20–84) without GERD symptoms were selected as controls: all of them showed normal endoscopy and pH-metry.

The study was conducted in accordance with the Declaration of Helsinki, was approved by our local Ethics Committee, and all enrolled subjects gave informed consent before participating in the study.

Endoscopy and biopsy samples

White light endoscopy was performed in all patients and controls, and the Los Angeles classification was used to grade esophagitis. A biopsy set was performed in each subject comprising two biopsies across the Z-line, and four biopsies (two for each site) at 2 and 4 cm above the Z-line. Since, as demonstrated previously [10], biopsies at 4 cm above the Z-line provide no additional information for the histological diagnosis of microscopic esophagitis that may not be obtained by samples at the Z-line and at 2 cm above it, these 4 cm biopsies were not evaluated in the present study.

All samples were formalin fixed, paraffin embedded, and stained with Hematoxylin & Eosin (H&E). The following parameters were analyzed: basal cell thickness for evaluation of BH, length of papillae for the evaluation of PE, presence of DIS, IE and neutrophils, erosions/healed erosions, and intraepithelial mononuclear cells. All evaluations were performed on a Nikon light microscope (Nikon Eclipse E600, Nikon Corporation, Japan) where a high-power field (HPF ×40) corresponds to an area of 0.66 mm^2.

BH, PE, and DIS were scored as 0 (absent), mild (1), and marked (2) on H&E. Criteria adopted to distinguish between normal and mild/marked lesions have already

been described [10, 13] and were thus applied. DIS was scored on the basis of their size and were considered 0, if absent, 1, if small, and 2, if large. IEs were counted in 3 HPFs (×40), and expressed as a mean value and scored as follows: 0 (absent), 1 (1 eosinophil), and 2 (>1 eosinophils). Intraepithelial neutrophils/erosions/healed erosions were scored as absent or present. Intraepithelial mononuclear cells were counted in 3 HPF (×40) and expressed as a mean value. All scoring was performed using a visual analogic scale composed of increasing severity of histopathologic lesions as previously published [10]. All these histological lesions were evaluated on all patient's biopsies (48 ERD, 59 NERD pH+, 12 NERD pH−) and controls (20 subjects) both at the Z-line and at 2 cm above it.

A global severity score (GSS—range 0–2), describing the overall severity of all assessed lesions, was calculated by adding the scores of the most informative lesions (namely BH, PE, DIS, and IE) and dividing the result by the number of assessable lesions [10]. If intraepithelial neutrophils, erosions or healed erosions were present, representing the most severe end of the spectrum, the assigned score was automatically 2. A cut-off value ≥0.35 was chosen as the most efficient in distinguishing patients from controls [10]. Histological assessment was performed by two expert pathologists (RF and LM) independently.

Immunohistochemistry

Immunohistochemistry was performed using the automated immunostainer *BenchMarkXT* (Ventana Medical Systems, Tucson, Arizona); 3,3′diaminobenzidine was used as chromogen.

The following antibodies were used:

- CD3 (SP7 clone, Immunomarkers Ventana, prediluted) for T lymphocyte evaluation;
- CD4 (SP35 clone, Cell Marque, dilution 1:10) for T helper lymphocyte evaluation;
- CD8 (SP47 clone, Cell Marque, prediluted) for T-suppressor lymphocyte evaluation;
- CD56 (123C3 clone, Cell Marque, prediluted) for natural-killer cell evaluation;
- CD68 (PG-M1, Diagnostic Biosystems, dilution 1:50) for macrophage evaluation;
- CD20 (L26 clone, Immunomarkers Ventana, prediluted) for B lymphocyte evaluation;
- CD138/syndecan-1 (B-A38 clone, Cell Marque, prediluted) for plasma cell evaluation;
- c-Kit (CD117 clone, Cell Marque, prediluted) for mast cell evaluation;
- S100 (4C4.9 clone, Immunomarkers Ventana, prediluted) for Langerhans cell evaluation.

Blinded immunohistochemical assessment was performed (by MB) by counting positive cells in the three most representative HPF (×40) and expressing as mean.

Statistical analysis

Quantitative variables were expressed as mean values and standard deviation (SD) as they were normally distributed (Shapiro–Wilk test). To analyze the differences among more than two groups, one-way ANOVA, with Scheffe multiple-comparison test correction, was used. Qualitative variables were summarized as counts and percentages and differences among groups were evaluated with Chi-square test. ROC analysis was used to find the optimal cut-off value of intraepithelial T lymphocyte (ITL)-count, capable of differentiating between controls and GERD patients. Spearman's rank coefficient (Rho) was used to test correlation between two study variables. All of the tests are double tailed and the limit of statistical significance was set to the commonly used 5 % ($p < 0.05$). Data analysis was performed with the software SATA (version: 13, Stata Corporation, College Station, 2013, Texas, USA).

Results

Intraepithelial B lymphocytes/plasma cells/NK cells/macrophages

The preliminary analysis of 30 cases (10 ERD, 10 NERD patients and 10 controls) both in Z-line and 2 cm biopsies, showed a complete absence of intraepithelial B lymphocytes in 81 % of cases. In the remaining 19 %, 1 (16 %) or 2 B lymphocytes (3 %) were demonstrated. No intraepithelial plasma cells, NK cells or macrophages were identified morphologically or using specific antibodies (CD138, CD56, and CD68, respectively). Scattered NK cells and macrophages were observed in the axis of the papillae and in the submucosa. No statistical difference was seen between ERD and NERD patients, between GERD patients and controls or between biopsy sites. B lymphocytes, often in nodular aggregates, plasma cells in the sub-mucosal layer (Fig. 1a), were seen both in GERD patients and controls, mainly in Z-line biopsies. The remaining cases were not stained or evaluated further.

Mast cells and Langerhans cells

The evaluation of mast cells by c-Kit staining and of Langerhans cells by S100 was performed on the entire case series and controls. Analysis was possible in all controls at

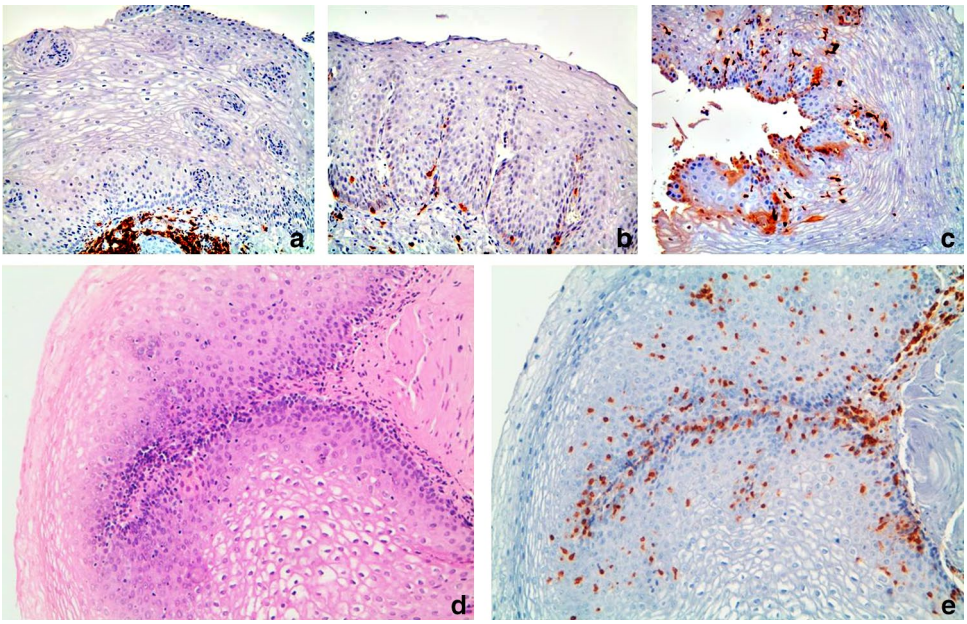

Fig. 1 **a** Nodular aggregates of B lymphocytes, in the sub-mucosal layer in a GERD patient; no evidence of intraepithelial B lymphocytes (Z-line biopsy, IHC, CD20 antibody, ×10 magnification); **b** intraepithelial mast cells around papillae and close to the basal layer in a GERD patient (Z-line biopsy, IHC, c-Kit antibody, ×10 magnification); **c** intraepithelial Langerhans cells located around papillae and close to the basal layer in a ERD patient (2 cm biopsy, IHC, S100 antibody, ×10 magnification); **d–e** comparison between H&E-stained and CD3-stained slides in evaluation of intraepithelial mononuclear cells/T lymphocytes (Z-line biopsy, H&E for **d**; IHC, CD3 antibody for **e**, ×10 magnification)

both biopsy sites for c-Kit and S-100. C-Kit staining was assessable in 107 GERD cases at the Z-line and also in 108 cases at 2 cm, while S-100 staining was assessable in 108 GERD cases at the Z-line and in 109 cases at 2 cm. Assessment was not possible due to section detachment or tissue depletion.

Intraepithelial mast cells were normally found in both Z-line and 2 cm biopsies of control subjects with a mean of 0.8 cells/HPF (Table 1) and were easily recognizable around papillae or close to the basal layer (Fig. 1b). Mast cells were also easily found in the fibro-vascular axis of papillae and in the sub-mucosal layer. No statistical significance was reached when comparing number of mast cells in ERD versus NERD versus controls both in Z-line biopsies and at 2 cm above it.

Langerhans cells were easily identified both in Z-line and 2 cm biopsies in control subjects with a mean value of 6.3 and 7.3, respectively, (Table 1) and were preferentially located around papillae and close to the basal layer (Fig. 1c). No statistical significance was noted when comparing number of Langerhans cells in patients (both ERD and NERD) versus controls. In contrast, the number of Langerhans cells in Z-line biopsies was significantly lower than the number detected in biopsies at 2 cm above the Z-line ($p = 0.0003$) in ERD patients but not in NERD or in controls.

Intraepithelial T lymphocytes

The evaluation of T lymphocytes by CD3 staining was performed on the entire case series and controls. Analysis was possible in all controls at both biopsy sites and in 108 GERD cases at the Z-line and in 110 cases at 2 cm; section detachment and tissue loss were the main reasons for non-assessment.

T lymphocytes present in vascular spaces, in the axis of papillae or in the sub-mucosal layer were excluded.

ITLs were found in all control subjects, both in Z-line and in 2 cm biopsies, with a mean value of 20.5 and 13.8 cells, respectively, (Table 1). ITLs were more numerous in the basal layer and around papillae but scattered cells were demonstrated in more superficial layers also. A significant increase of ITLs was seen both in Z-line and in 2 cm biopsies in all GERD, ERD, and NERD patients when compared to controls (p between 0.0041 and >0.0001). No differences, however, were noted when comparing NERD with ERD patients, whether in Z-line biopsies or at 2 cm ($p = 0.16$ and $p = 0.22$, respectively).

With respect to biopsy site, a significantly higher number of ITLs was demonstrated in GERD patients at the Z-line compared to 2 cm ($p < 0.0001$). The number of mononuclear cells evaluated in H&E-stained slides and the number of ITLs evaluated in CD3-stained slides were significantly

Table 1 Distribution of inflammatory cells by site and categories of patients/controls

Categories	Site	*N*	Mast cells			Langerhans cells			T lymphocytes*		
			Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
ERD	2 cm	40	1.5	1.7	0–10	7.3	3.1	2–15	45.6	27.7	7–142
	Z-line	42	1.6	1.0	0–4	5.1	2.6	2–11	55.2	29.6	17–134
NERD pH+	2 cm	57	1.4	2.7	0–18	7.0	4.0	1–20	43.9	30.9	8–200
	Z-line	57	1.6	1.5	0–6	5.7	3.2	1–15	53.4	37.1	9–170
NERD pH-	2 cm	11	1.5	1.2	0–3	7.9	4.4	3–16	51.7	34.1	10–109
	Z-line	11	1.1	0.8	0–2	6.4	4.0	2–14	51.1	38.5	119–122
All patients	2 cm	108	1.5	2.1	0–18	7.1	3.7	1–20	42.4	29.1	7–200
	Z-line	110	1.5	1.2	0–6	5.4	3.0	1–15	51.7	33.3	9–170
Controls	2 cm	20	0.8	0.7	0–2	7.3	4.8	2–15	13.8	9.3	3–39
	Z-line	20	0.8	1.0	0–3	6.3	2.9	3–12	20.5	17	4–59

ERD erosive reflux disease, *NERD pH+* non-erosive reflux disease with abnormal pH-metry, *NERD pH−* non-erosive reflux disease with normal pH-metry

* Both at Z-lines and 2 cm, all comparisons between controls and patients (ERD, NERD pH+, NERD pH−) were significant ($p < 0.01$). Differences within patient categories were not significant

correlated ($p < 0.0001$). The number of mononuclear cells was always lower than the number of ITLs (mean of 14 mononuclear cells vs 47 T lymphocytes in Z-line biopsies and 12 vs 30 in biopsies at 2 cm above Z-line); this may be due to the difficulties in clearly identifying intraepithelial mononuclear inflammatory cells in H&E-stained slides (Fig. 1d, e).

ROC analysis applied to the ITL count demonstrated that a cut-off value of 20 CD3-positive cells/HPF can distinguish GERD patients from controls with good sensitivity and specificity (respectively: 89.5 and 75 % in biopsies at Z-line; 85.5 and 85 % in biopsies at 2 cm above Z-line—Fig. 2). On this basis, ITL count was categorized as normal when ≤20/HPF and pathologic when >20/HPF (Table 2). The overall sensitivity (i.e., presence in patients) was 88.8 %, whereas specificity (i.e., absence in controls) was 80 %.

Finally, the correlation between ITL count and other histological lesions as well as the GSS was considered. Data (Table 3) show a significant correlation between ITL count and all other evaluated histological lesions, including the GSS, except for DIS in biopsies at 2 cm above Z-line.

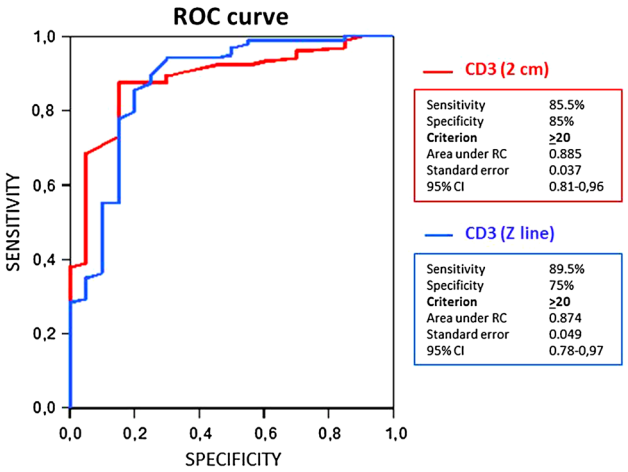

Fig. 2 ROC curve analysis of ITL count obtained with CD3 antibodies in biopsy samples taken at 2 cm (*red*) and at the Z-line (*blue*) in all patients and controls

Intraepithelial T lymphocyte subpopulations (CD4 and CD8+)

In a subset of cases, the distribution of CD4+ and CD8+ T lymphocytes was evaluated. The analysis was possible in 20 ERD cases, 20 NERD cases, and 10 controls which had sufficient amount of tissue available in the paraffin block.

A significant difference was seen in CD4+ and CD8+ T lymphocytes between biopsies from the Z-line and 2 cm above it in ERD patients ($p < 0.001$ and 0.005 respectively), while no significant difference was seen in NERD patients nor controls even though mean value was always lower at 2 cm compared to the Z-line.

CD4+ T lymphocytes were found in control subjects (mean value at Z-line: 11.5; mean value at 2 cm: 8.8), NERD cases (mean value at Z-line: 27.4; mean value at 2 cm: 23.2), and ERD cases (mean value at Z-line: 33.8; mean value at 2 cm: 24.5) with a significant increase in ERD and NERD patients versus controls (p between 0.002 and 0.006) but with no differences between ERD and NERD patients (p between 0.26 and 0.79).

CD8+ T lymphocytes were found in control subjects (mean value at Z-line:10.4; mean value at 2 cm: 8.5),

Table 2 Prevalence of intraepithelial T lymphocytes (ITLs) >20 in patients and controls

Group	No. of cases at 2 cm	>20 ITLs at 2 cm	%	No of cases at Z-line	>20 ITLs at Z-line	%
ERD	42	38	91	40	38	95
NERD pH+	57	46	81	57	50	88
NERD pH−	11	10	91	11	9	82
All patients	110	94	85.5	105	94	89.5
Controls	20	3	15	20	5	25

Both at Z-lines and 2 cm, all comparisons between controls and patients (ERD, NERD pH+, NERD pH−) were significant ($p < 0.001$). Differences within patient categories were not significant

ERD erosive reflux disease, *NERD pH+* non-erosive reflux disease with abnormal pH-metry, *NERD pH−* non-erosive reflux disease with normal pH-metry

Table 3 Correlation between intraepithelial T lymphocytes (ITLs) and other histological lesions/global severity score by site

	ITLs 2 cm	ITLs Z-line
Basal cell hyperplasia ($N = 128$)	Rho = 0.39	Rho = 0.28
	$p < 0.0001$	$p = 0.003$
Papillae elongation ($N = 115$)	Rho = 0.20	Rho = 0.38
	$p = 0.033$	$p < 0.0001$
Dilatation of intercellular spaces ($N = 129$)	Rho = 0.15	Rho = 0.28
	$p = 0.19$	$p = 0.003$
Intraepithelial eosinophils ($N = 130$)	Rho = 0.23	Rho = 0.23
	$p = 0.008$	$p = 0.008$
Global severity score ($N = 130$)	Rho = 0.38	Rho = 0.20
	$p < 0.0001$	$p = 0.032$

NERD cases (mean value at Z-line: 24.3; mean value at 2 cm: 21), and ERD cases (mean value at Z-line: 27.7; mean value at 2 cm: 21.3) with a significant increase in ERD and NERD patients versus controls (p between 0.002 and 0.008) but with no differences between ERD and NERD patients (p between 0.54 and 0.94).

CD4+ T lymphocytes were 53.2 % (range 52.5–55.8 %) and CD8+ T lymphocytes were 46.8 % (range 44.2–47.5 %) of all CD3 lymphocytes. No statistical difference in proportion in CD4+/CD8+ was seen between controls, NERD and ERD patients both at the Z-line and at 2 cm above it.

Discussion

The histologic diagnosis of GAME has been a matter of discussion for numerous years. It was initially based on the presence of inflammatory cells, and the role of neutrophils and eosinophils was clearly demonstrated starting from the 1960s [30–32]; their importance in the histologic diagnosis of GAME has been recently reappraised considering that their prevalence is low and they are almost always found in ERD patients, in which the histological diagnosis is clinically irrelevant. On the other hand, the role of intraepithelial mononuclear cells (lymphocytes, mast cells, and Langerhans cells) is less clear and still debated.

Mast cells

Animal models have suggested that the presence of intraluminal acid in the esophagus is associated with degranulation of mast cells and a role for this event has been suggested in the pathogenesis of reflux disease [24]. The number of mast cells in fact increases both in esophagitis and, more dramatically, in Barrett's esophagus [26, 27, 33]. Recently, the key role of mast cells in recruiting neutrophils in acid-induced damage to squamous epithelium [25] has been demonstrated in a mouse model. Furthermore, the interaction between mast cells and eosinophils has been suggested in the pathogenesis of eosinophilic esophagitis where mast cell intraepithelial count seems useful in differentiating this condition from reflux [34]. Our results show that in GERD patients there is a slight increase of mast cells with respect to controls, but this finding is not statistically significant. So far the presence of mast cells has not proven to be a useful parameter for diagnosing GERD.

Langerhans cells

The identification of Langerhans cells in esophageal squamous epithelium by electron microscopy dates 1976 [35] and this finding was later confirmed in immunohistochemistry [23]. Our observations confirm the presence of Langerhans cells in both controls and in GERD patients, with no significant difference between groups. Langerhans cell count is therefore of little use in GERD diagnosis.

Lymphocytes

Several authors [17–20] demonstrated that lymphocyte aggregates in the lamina propria were aspecifically present in controls and in symptomatic patients. Geboes [23]

demonstrated the presence of scattered intraepithelial T cytotoxic lymphocytes with an immune barrier function, while T helper and B lymphocytes were commonly located in the lamina propria. Furthermore, only T cytotoxic lymphocytes increased in esophagitis, while no differences were noted in lamina propria inflammatory cells. In contrast, other studies did not demonstrate ITL increase to be either an independent diagnostic parameter [21, 22] or that this correlated with other histological lesions [36]. More recently, ITLs have been shown to be more frequently detected in more distal biopsies (Z-line vs 2 cm above it) and in ERD patients [37]. Our results confirm such findings. In particular, while the presence of B lymphocytes in esophageal epithelium seems to be occasional, ITLs are normally found and they increase in GERD patients although their increase with increasing severity of LA classification has been shown to be modest [38]. The higher value of ITLs found at the Z-line as compared to 2 cm above it, probably reflects the already demonstrated [10] higher severity and frequency of all GERD-related lesions at the Z-line versus 2 cm above it. A cut-off value of 20 ITLs can distinguish between control and GERD patients (both ERD and NERD) with good sensitivity and specificity. No significant difference in ITLs was demonstrated between ERD and NERD patients. As with other histological lesions contributing to the GSS, a high sensitivity and specificity was demonstrated for ITL count in both ERD and NERD patients, leading to the hypothesis that NERD and ERD are part of the spectrum of GERD disease [10, 11, 39]. Finally, ITL count significantly correlates with other histological parameters and the GSS, so demonstrating its possible use in the histological diagnosis of GAME. However, debate is still open on some points and in particular are the following: (1) histology has an unclear role in GERD diagnosis and as yet its use is still not recommended and limited to few cases not responsive to PPI therapy or with atypical symptoms and negative endoscopy; (2) the use of immunohistochemistry for ITL assessment leads to an additional cost. For these reasons, it seems reasonable to suggest a possible use of ITL count, solely in the context of an experimental diagnostic setting for GERD and GERD-related procedures or treatment.

Our aim is focused on trying to resolve the well-known dispute whether ITL count could be an additional parameter useful in routine diagnosis of GAME. Several authors have focused on subtyping T cells colonizing esophageal squamous epithelium, demonstrating that most esophageal ITLs exhibit a cytotoxic phenotype [40], while the significance of CD4+/CD8+ T lymphocyte ratio is poorly understood [41, 42]. In the present study, no significant difference in CD4+ or CD8+ T lymphocyte distribution with regards to neither biopsy site nor disease subset (NERD, ERD)/controls was observed. Our CD4+/CD8+ ratio is roughly of 1:1; previous reports refer a predominance of CD4+ lymphocytes in GERD children [42], while an inverse CD8+ predominance is seen in lymphocytic esophagitis [41]. A limit to the use of formalin-fixed paraffin-embedded biopsy material to identify lymphocyte subpopulations must be stressed. This is mainly due to the scarcity of tissue and the need to perform different antigen–antibody reactions (CD20, CD3, CD4, etc.) on serial sections. More appropriate techniques could be double immunostaining or cytofluorometry.

Finally, lymphocytic esophagitis has been recently described as an emerging condition, clinically different from GERD, characterized by a significant amount of intraepithelial lymphocytes with spongiosis in the absence of significant neutrophilic or eosinophilic infiltrates [43]. Other authors [44], however, query the possibility that lymphocytic esophagitis is a separate entity and suggest that this condition may represent "an extreme in the spectrum of GERD". Reviewing all esophageal biopsies of our series, only one case showed the above mentioned diagnostic criteria in H&E but with GERD-related features (typical reflux symptoms and pathologic pH-metry), confirming the rarity of the disease and suggesting a possible overlap with GERD.

In conclusion, a better understanding of the inflammatory cells involved in GERD may lead to new insights into the pathophysiology of this extremely common and variegated disease.

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Statement of Human Rights All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

Conflict of interest Luca Mastracci, Martina Bruzzone, Elena Pacella, Carmine Tinelli, Patrizia Zentilin, Edoardo Savarino, Annalisa De Silvestri, Roberto Fiocca, and Federica Grillo declare that they have no conflict of interest.

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