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Characterization of Immune Cell Populations and Acid-Sensing Receptors in the Human Esophagus

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Abstract: Introduction: Esophageal inflammatory diseases are frequent diagnoses in clinical practice and have diverse etiologies, the most common being those associated with the exposure to gastric content, drugs and allergens. In diseases, the immunological component is well identified in endoscopic biopsies, which mainly contain the epithelium and the *lamina propria*; however, deeper layers are less studied. Moreover, the esophageal capacity of sensing luminal compounds is poorly understood. Methods: In transmural sections from proximal, middle and distal esophagus obtained from deceased patients, we performed a phenotypic analysis of the main immune cell populations and acid-sensing receptors by immunohistochemistry and immunofluorescence methods. Results: A total of nine donors were studied (absence of pathology, optimal tissue preservation and orientation). We found the following: (1) the vascular *papillae* and the *lamina propria* are the most infiltrated layers by the lymphoid lineage (T and B lymphocytes), followed by the epithelium, while the smooth muscular layers are mainly populated by the myeloid lineage (macrophages and mast cells); (2) intraepithelial macrophages are consistently found along the esophagus; and (3) eosinophils are absent in all the esophageal layers. The acid-sensing receptors ASIC-1, ASIC-2 and δENAC are expressed in the esophageal epithelium and in the *lamina propria*, yet only ASIC-2 is expressed in the *muscularis mucosae*. Conclusions: The human esophagus contains a differential distribution of immune cells and acid-sensing receptors across its layers. This study extends the esophageal histological knowledge previously described and reinforces its role as a defensive and sensing organ.

Keywords: esophagus; immune system; acid-sensing receptors

1. Introduction

Despite a seemingly simple appearance and function, the esophagus is an essential component of the gastrointestinal tract. While being the only segment lacking digestive and absorptive activity, the esophagus hosts a complex neuromuscular structure with the crucial function of allowing the propulsion of the bolus from the mouth to the stomach. Additionally, it develops an efficient defensive barrier by the epithelium and the immune components that populate this organ.

In healthy humans, the esophageal lining contains a non-keratinized stratified squamous epithelium of around 30 cell layers [1], distributed into the stratum corneum, stratum spinosum, or the prickle cell layer, and stratum germinativum. The stratum corneum, or the functional layer, comprises the outermost cell layers and acts as a mechanical barrier for protection against luminal components [1]. The epithelial cells establish a tight physical barrier, in which the basal layer proliferates and moves upwards, constantly replacing the lining of the epithelium. Unlike other parts of the gastrointestinal tract, the esophageal epithelial layer lacks mucus-producing cells and endocrine cells, but develops a fundamental barrier function, restricting the entry of antigens and other luminal contents into the organism by different protective mechanisms [1].

In esophageal diseases of different etiology, a significant increase in immune cells is found in the epithelium, presumably challenging the epithelial integrity. The most common esophageal diseases showing immune infiltration include the gastroesophageal reflux disease (GERD), eosinophilic esophagitis (EoE), lymphocytic esophagitis (LE) and motility disorders such as ineffective esophageal motility and achalasia [2–6]. The most frequent esophageal disease is GERD, with an estimated worldwide prevalence of 8–33% [7]. Thirty percent of these patients have erosive esophagitis, characterized by infiltration of lymphocytes, eosinophils and mast cells in the *lamina propria* and the epithelium [8,9]. Nevertheless, the majority of GERD patients present with a normal esophagus at endoscopy, and they are diagnosed as having non-erosive GERD. A large proportion of patients with GERD experience motility disorders such as ineffective esophageal motility, esophagogastric junction outflow obstruction, absence of peristalsis and achalasia [10]. The cause of this altered motility is unknown, but some evidence suggests the participation of myenteric and muscular inflammation [11].

Several mechanisms have been implicated in the pathophysiology of GERD and esophagitis. Among them, hypersensitivity is a common, often returning, observation in all GERD patients despite the presence or absence of erosions [12]. Visceral hypersensitivity is a relevant concept in symptom generation. Yet, the characterization of the receptors responsible for this hypersensitivity and their sensing spectrum has not been fully elucidated in the human esophagus. Acid perception in the esophagus has been attributed mainly to the TRPV1 receptors [13]; nevertheless, other possible candidates such as acid sensing receptors (ASIC) and the delta subunit of the epithelial sodium channel (δENaC) have not been explored.

Despite the increasing prevalence of esophageal disorders and the discovery of new inflammatory entities of this organ, the specific role of resident immune populations in their pathophysiology is not yet known [14,15]. This is attributed, at least in part, to the limited area of the tissue usually available, commonly endoscopic biopsies, mainly containing the esophageal epithelium and to a lesser extent the *lamina propria*. Scarce information is available on deeper layers, which may be critical to understand the pathophysiology of motility disorders. The immune cell populations better described are eosinophils [16] and lymphocytes [17], and to a lesser extent mast cells and macrophages.

Therefore, the specific aim of this study was to characterize the general immune populations and the acid-sensing receptors, as well as their distribution throughout the

different layers and along the length of the adult human esophagus in health. A detailed description of the esophageal immune populations and of the sensing receptors may contribute to better discriminating between health and disease, and to precisely establishing the histological definition of newly recognized entities.

2. Material and Methods

2.1. Biological Samples

Esophageal tissue was obtained from deceased patients from Hospital de la Santa Creu i de Sant Pau (Barcelona, Spain) and from Leuven University Hospital (Leuven, Belgium), and the samples were processed in the Digestive Diseases Research Unit of the Hospital Vall d'Hebron. This study was carried out in accordance with the guidelines of the Declaration of Helsinki and the principles of good clinical practice, and the research protocol was approved by the Ethics Committee at Hospital Vall d'Hebron [PR(AG)94/2013] and the local Ethical Committee of the University of Leuven (S56978-ML10868). Tissue sections were taken during the autopsy for histological examination at maximum 6 h after decease. Inclusion criteria were the following: (1) absence of gastrointestinal pathologies (by clinical history), (2) absence of pathological findings during histological examination. From each donor, transmural sections from proximal, middle and distal esophagus (classification made due to the differential musculature) were obtained and fixed in 4% paraformaldehyde for 24 h, dehydrated and embedded in paraffin, following standard procedures.

2.2. Tissue Collection

The entire esophagus was procured in block, together with the stomach. The proximal esophagus was macroscopically identified as it is composed of purely striated (skeletal) muscle. The upper 5% to 33% of the muscularis propria is composed exclusively of striated muscle, and the distal part is composed of smooth muscle. In between, the area where the striated and smooth muscle intermix is called the transition zone [18]. Then, a transmural piece of 2 cm was collected 3 cm orally to the transition zone of the esophagus (proximal esophagus). A piece of 2 cm was obtained from the distal esophagus 0.5–1 cm above the esophago-gastric junction (distal esophagus). Finally, a piece of 2 cm of the middle part of the remaining segment was collected (middle esophagus).

2.3. Histological Procedures

Transmural tissue sections were cut at 5 µm, placed on microscope slides and de-paraffinized in xylene, gradually rehydrated by using decreasing concentrations of ethanol. Samples were stained with hematoxylin and eosin (H&E), and histological examination was performed by an experienced pathologist. Only samples with optimal orientation (all esophageal layers visible) were selected for subsequent analysis.

2.3.1. Immunohistochemistry

Samples were dehydrated and processed for heat-induced epitope retrieval (HIER) in citrate buffer at pH6 or tris-EDTA at pH9 buffer for 10 min at 120 °C. Samples were washed in 0.01 M PBS (Sigma, St. Louis, MO, USA) after each step. Endogenous peroxidase activity was inactivated by adding Peroxidase-Blocking Solution (K4007/K4010, Agilent, Santa Clara, CA, USA), and nonspecific unions were avoided by adding Protein Block Serum-Free (X0909, DAKO, Glostrup, Denmark). The immune populations identified based on surface markers were the following: B lymphocytes (CD20), T lymphocytes (CD3), T helper lymphocytes (CD4), T cytotoxic lymphocytes (CD8), macrophages (CD68), mast cells (tryptase) and eosinophils (MBP). The acid-sensing receptors studied were acid-sensing ion channels type 1 (ASIC-1), acid-sensing ion channels type 2 and the delta subunit of the epithelial sodium channel (δENaC). Primary antibody was added, diluted in antibody diluent (Dako) (conditions detailed in Table S1). Positive staining was revealed after the incubation with the secondary antibody (Table S2) followed by the staining with the peroxidase substrate 3,3′-diaminobenzidine tetrachloride (K4007/K4010, Agilent, Santa

Clara, CA, USA). Slides were counterstained with 50% hematoxylin, dried and observed under an optical LEICA DMBL microscope (Wetzlar, Germany), coupled with an OLYMPUS DP26 video camera.

2.3.2. Immunofluorescence

Similarly, samples were dehydrated and processed for HIER in a citrate buffer and washed in PBS. Permeabilization was performed with 0.4% Triton and 0.2% Tween 20, and Protein Block Serum-Free Ready-to-use (Dako) was used to avoid nonspecific unions. The acid-sensing receptors studied were the acid-sensing ion channels type 2 and the delta subunit of the epithelial sodium channel (δENaC). Primary and secondary antibodies were diluted in blocking serum (Tables S1 and S2, respectively) and nuclei were counterstained using 4′, 6-Diamidino-2-phenylindole (DAPI) (Sigma). Slides were cover-slipped and mounted with ProLong Gold Antifade reagent (Molecular Probes, Madrid, Spain) and kept in the dark at 4 °C until analysis under a fluorescence microscope. Images were acquired and quantified in a blinded manner using the Olympus XM10 camera and the Olympus cellSens Entry 1.16 software (Olympus, Barcelona, Spain).

2.4. Quantitative Analysis of Immune Cells

Immune cell counting was performed in a blinded manner in micrographs obtained in at least 10 non-overlapping fields using the CellSens Standard 1.7 software. Positive staining for each immune cell type was identified in the different layers of the esophageal sections. The area of each layer was calculated with the software, and the number of positive cells were counted and expressed as follows: the mean cellular density (C_ρ) was estimated as $C_\rho = \sum(Q/A)$, where Q is the total number of cells counted, and A is the area determined for each esophageal layer. The results are expressed as the number of cells per area, in mm^2. No reliable counts could be performed in the submucosal layer due to tissue discontinuity after the immunostaining procedures.

2.5. Statistical Analysis

Data are expressed as mean ± SD or median (range). Normality of data distribution was tested by the D'Agostino and Pearson omnibus tests. Outliers were identified by the Grubb's test. Normally distributed data were compared by One-Way ANOVA. Data not following normality were compared by the Kruskal–Wallis test. Multiple comparisons were corrected using Dunn's multiple comparisons test (non-parametric) or Tukey's multiple comparison test (parametric). Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Study Population

A total of 18 donors were included in this study, and the transmural sections collected from each esophagus were carefully analyzed. The histological evaluation of the samples identified sections from 10 donors with proper orientation in each tissue layer for subsequent analysis. The presence of dilated intercellular spaces and basal cell hyperplasia led the pathologist to discard the specimens from 1 donor as a histological diagnosis of GERD. A total of nine donors were finally included in the analysis. The donors' mean age was 57 ± 11 years old with a male/female distribution of 6:3. Most common cause of death was a cerebrovascular accident (three); other causes were pulmonary hemorrhage (one), heart failure (one), pneumonia (one), myocardial infarction (one), amyotrophic lateral sclerosis (one) and complications during cranial surgery (one).

3.2. Structural Analysis

All samples showed histological characteristics of the esophageal tissue (Figure 1A–C): a normal non-keratinized stratified squamous epithelium supported by connective tissue, the *lamina propria*, mainly constituted by collagenous and elastic fibers. The *vascular papillae* were identified as extensions of the *lamina propria* within the epithelium for up to two thirds

from the base of the epithelium. In all esophageal portions (proximal, middle and distal), lymphoid aggregates and scattered leukocytes were identified, especially in the epithelium and in the *lamina propria*. The *muscularis mucosae* layer was identified, discontinuous, and in between the *lamina propria* and the submucosa layer. Sero-mucosal glands were observed in the submucosa region, and the *muscularis externa* was the thickest layer in all esophageal regions. Differences were observed when comparing the esophageal regions of the *muscularis externa:* entirely striated muscle in the proximal esophagus, mixed (smooth and striated muscle) in the middle and completely smooth in the lower portion.

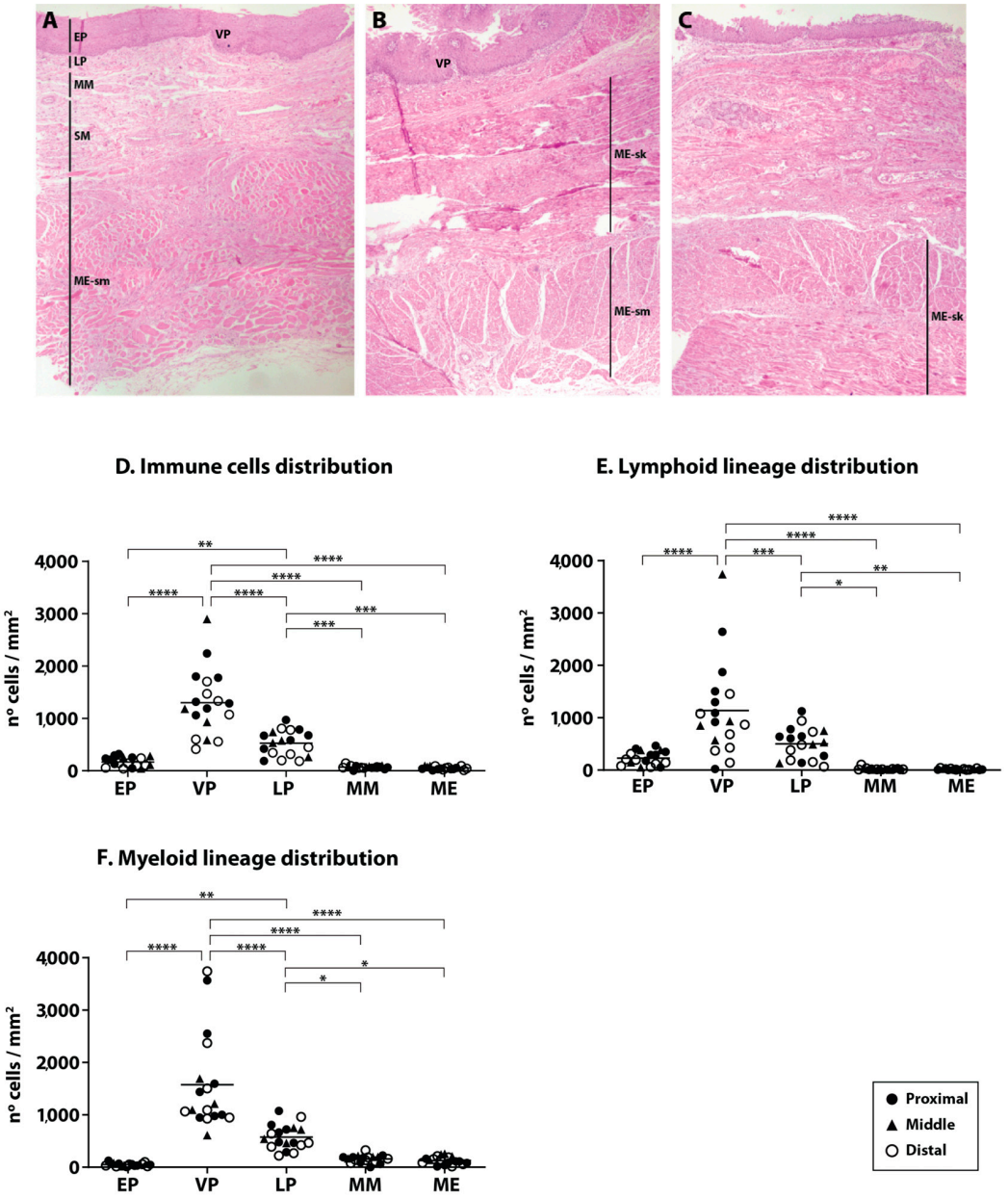

Figure 1. Esophageal anatomical layers and distribution of immune cells within the human esophagus. Representative images of the proximal (**A**), middle (**B**) and distal (**C**) esophageal histological structure. The different layers are indicated as EP (epithelium), VP (*vascular papillae*) LP (*lamina propria)*, MM (*muscularis mucosae*), SM (submucosa), ME (*muscularis externa*), sm (smooth muscle) and sk (skeletal muscle). (**D**) Distribution of immune cells. Each individual represents the average of all the immune cells, analyzed based on the surface markers (CD3, CD4, CD8, CD20, CD68, tryptase and MBP). (**E**) Lymphoid lineage distribution. Each individual represents the average of all the immune cells analyzed from the lymphoid lineage (CD3, CD4, CD8 and CD20). (**F**) Myeloid lineage distribution.

Each individual represents the average of all the immune cells analyzed from the myeloid lineage (CD68, tryptase and MBP). Normally distributed data were compared by One-Way ANOVA. Data not following normality were compared by Kruskal–Wallis test. Multiple comparisons were corrected using Tukey's multiple comparison test (parametric) or Dunn's multiple comparisons test (non-parametric). (* $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$, **** $p < 0.0001$.).

3.3. Distribution and Phenotype of Immune Cells in the Esophagus

The different immune cell populations were identified in the esophageal anatomical layers observed (epithelium, *vascular papillae*, *lamina propria*, *muscularis mucosae* and *muscularis externa*) within the proximal, middle and distal esophagus, after each immunostaining procedure (Figures 1, 2 and S1). Positive controls of the stainings are shown in Figure S2.

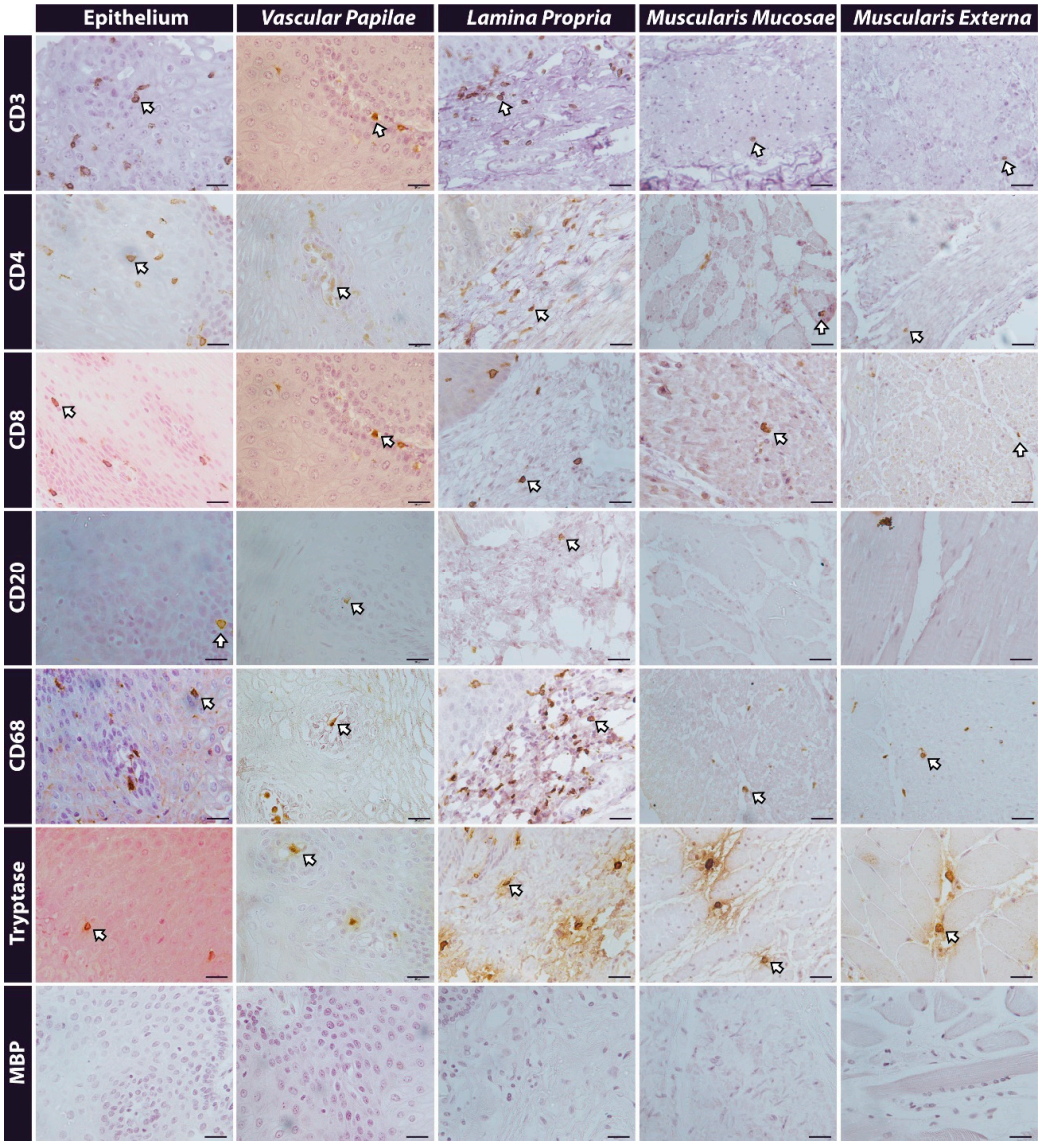

Figure 2. Representative images of immunostainings for each immune cell population analyzed in different regions and anatomical layers of the esophagus. The represented results are the average of the 3 esophageal regions. In each image, arrowheads point at positive staining (magnification 400×; scale bar 20 microns).

The analysis of the immune cell populations revealed a heterogeneous distribution of leukocytes across the different esophageal tissue layers. The most populated layers were the *vascular papillae* (1302 ± 628 cells/mm^2) and the *lamina propria* (528 ± 244 cells/mm^2), as compared to the epithelium (169 ± 94 cells/mm^2), *muscularis mucosae* (71 ± 30 cells/mm^2) and *muscularis externa* (54 ± 29 cells/mm^2) (Figure 1D). The lymphoid lineage, containing B and T lymphocytes, was present in all layers, but it was more abundant in the epithelium (1228 ± 135 cells/mm^2), *vascular papillae* (925 (22–3741) cells/mm^2) and *lamina propria* (503 ± 304 cells/mm^2) compared to the *muscularis mucosae* (14 (0–105) cells/mm^2) and *muscularis externa* (16 ± 13 cells/mm^2) (Figure 1E). The myeloid lineage, including macrophages and mast cells, was also identified in all layers, and was more abundant in the *vascular papillae* (1154 (610–3741) cells/mm^2) and the *lamina propria* (575 ± 236 cells/mm^2), compared to the rest of the esophageal layers (epithelium: 51 ± 30 cells/mm^2, *muscularis mucosae:* 159 ± 73 cells/mm^2 and *muscularis externa*: 129 ± 74 cells/mm^2) (Figure 1F). The distribution of immune cells was similar between the three esophageal regions (Figure 1D–F), and no differences were identified due to the sex or age of the donors. Notably, eosinophils were not identified in any esophageal layer.

Within the epithelium, the immune cells were mainly located in the basal and parapapillary areas. The T lymphocyte population (CD3$^+$, including both subtypes CD4$^+$ and CD8$^+$) was the most abundant leukocyte type infiltrating the epithelium, as compared with the other cells analyzed. The epithelium also contained macrophages and mast cells and a few B lymphocytes (Table 1 and Figure 3). No statistical difference was identified between the proximal, middle and distal esophageal regions (Table 1).

Table 1. Immune cell counts in the different layers and regions of the esophagus. For each anatomical esophageal layer, the number of cells per area is indicated (cells/mm^2). Data are expressed as median (range).

Epithelium						
cell marker	**CD3**	**CD4**	**CD8**	**CD20**	**CD68**	**Tryptase**
Proximal	403 (131–968)	444 (17–1176)	350 (47–548)	4 (0–8)	76 (13–244)	22 (4–56)
Middle	418 (142–689)	260 (11–580)	260 (94–633)	2 (0–27)	78 (15–156)	15 (8–22)
Distal	229 (95–315)	234 (12–362)	130 (20–616)	0 (0–13)	61 (1–120)	22 (4–79)
Vascular papillae						
cell marker	**CD3**	**CD4**	**CD8**	**CD20**	**CD68**	**Tryptase**
Proximal	2008 (1190–2826)	2266 (0–8452)	278 (0–767)	294 (0–924)	1049 (486–2021)	1272 (904–5481)
Middle	994 (615–1414)	2057 (304–13,999)	433 (0–601)	0 (0–350)	1747 (1120–2193)	202 (0–1907)
Distal	478 (0–3373)	1183 (12–2608)	497 (0–1833)	0 (0–280)	1430 (198–2297)	1581 (25–5186)
Lamina propria						
cell marker	**CD3**	**CD4**	**CD8**	**CD20**	**CD68**	**Tryptase**
Proximal	749 (283–1650)	1130 (16.5–1592)	264 (153–1459)	96 (0–253)	846 (132–1835)	350 (142–596)
Middle	601 (70–947)	1108 (134–1631)	222 (121–290)	166 (0–330)	928 (604–1186)	330 (252–391)
Distal	698 (53–1028)	623 (99.5–2239)	202 (108–703)	29 (0–100)	414 (70–1338)	392 (214–587)
Muscularis mucosae						
cell marker	**CD3**	**CD4**	**CD8**	**CD20**	**CD68**	**Tryptase**
Proximal	0 (0–95)	0 (0–144.5)	5 (0–16)	0 (0–0)	123 (93–183)	245.5 (0–350)
Middle	13 (0–22)	43 (0–67.5)	19 (8–31)	0 (0–0)	187 (132–228)	206 (151–266)
Distal	41 (0–114)	24 (0–2239)	2.5 (0–32)	0 (0–0)	174 (38–357)	149.5 (80-5–294)

Table 1. *Cont.*

Muscularis propria						
cell marker	**CD3**	**CD4**	**CD8**	**CD20**	**CD68**	**Tryptase**
Proximal	0 (0–22.5)	6.5 (0–21)	9 (0–142)	0 (0–8)	72 (10–294)	65 (0–216)
Middle	6.5 (0–14)	6.5 (0–33)	57.5 (18.5–89.5)	0 (0–0)	258 (27.5–356)	208 (135–252)
Distal	9 (0–46)	19 (0–123)	29 (5–81)	0 (0–4.5)	102 (12.5–342)	85 (20–351)

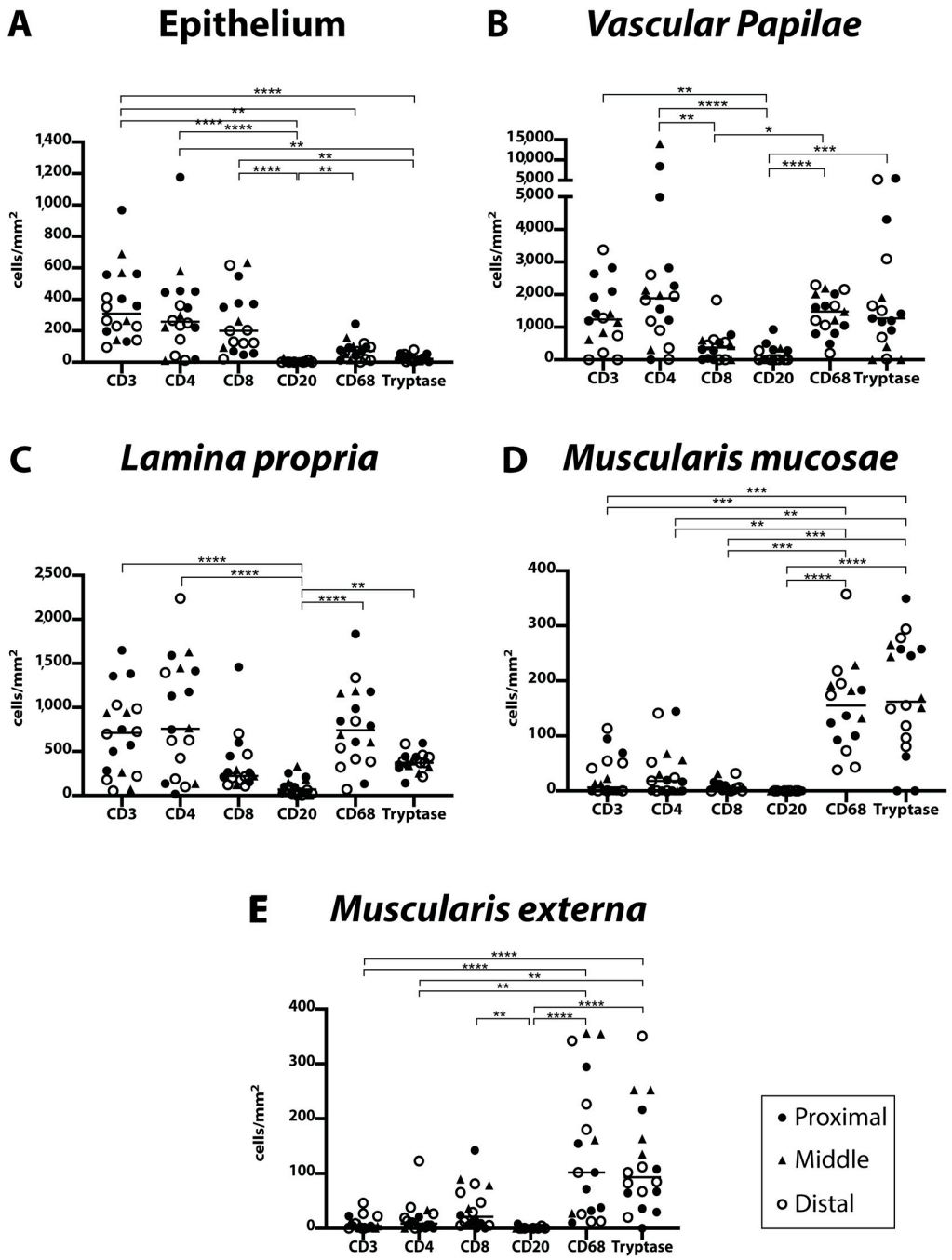

Figure 3. Distribution of immune cell populations in each esophageal layer. Quantification of each immune cell population within the epithelium, *vascular papillae*, *lamina propria*, *muscularis mucosae* and *muscularis externa* for each esophageal region (proximal, middle, distal). The Kruskall–Wallis test was performed for every cell type comparison. Dunn's multiple comparison test was used for adjusted *p*-value calculations (* $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$, **** $p < 0.0001$).

The *vascular papillae* contained the largest proportion of immune cells in the esophagus. The most abundant cell type was the T lymphocyte population, with the CD4 T cell subtype being the predominant one (Table 1; Figure 3). No differences were identified in the number of cells between the esophageal regions analyzed (Table 1). The *lamina propria* contained all the cell types studied, with T lymphocytes (CD4 subtype) and macrophages being the most abundant ones (Table 1, Figure 3).

The muscular layers, both the *muscularis mucosae* and the *muscularis externa*, were characterized by the myeloid lineage infiltration of mast cells and macrophages, small amounts of T lymphocytes and near absence of B lymphocytes (Figure 3). No statistical difference was identified between the proximal, middle and distal esophageal regions (Table 1). The distribution of every immune cell type analyzed among the anatomical layers, as well as their statistical differences, is shown in Figure S3.

3.4. Distribution of Acid-Sensing Receptors in the Esophagus

The distribution of acid-sensing receptors was analyzed within the distal third of the esophagus. ASIC-1 staining was localized on the plasma membrane of the functional layer of the esophageal epithelium, indicating that ASIC-1 receptors are expressed in epithelial cells (Figure 4). ASIC-1 immunoreactivity was clearly visible very close to the basal layer of the epithelium and in deeper areas of the *lamina propria*, including the *vascular papillae* (Figure 4), likely to be ASIC1-positive sensory nerve fibers. No positive staining was observed in the *muscularis mucosae*. In the absence of the primary antibody (ASIC-1), no immunoreactivity was found.

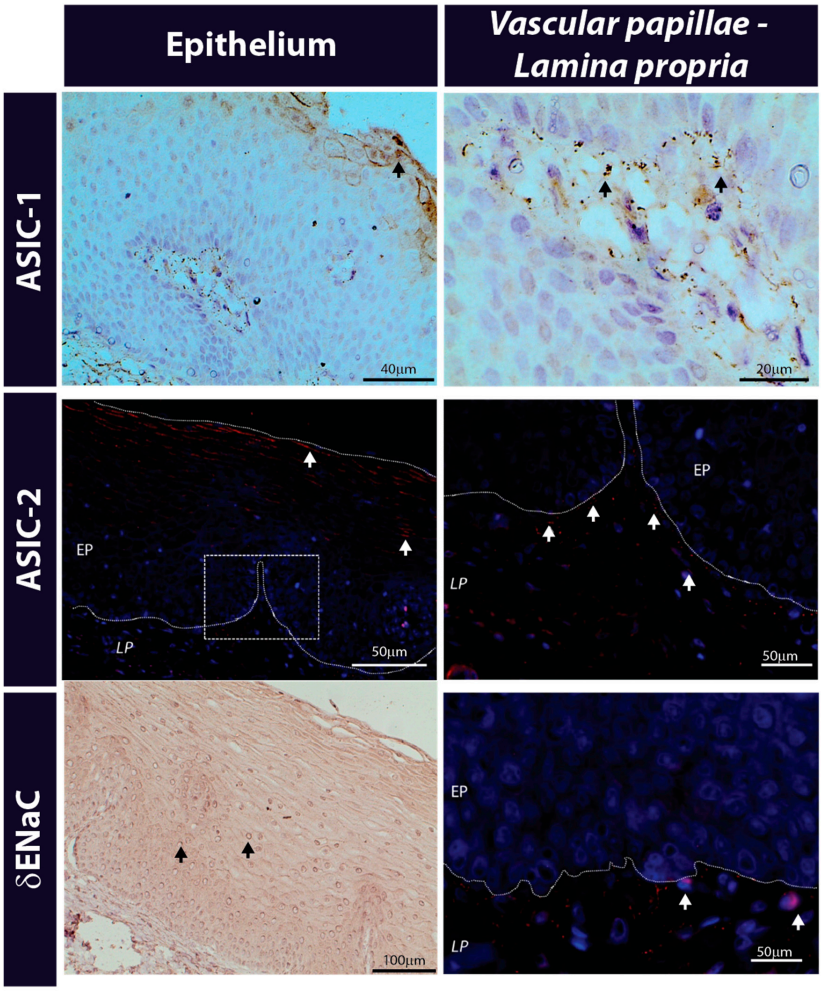

Figure 4. Acid-sensing receptors in the human esophagus. Representative images of the ASIC-1 staining, observed in the functional layer of the epithelium (external) and under the basal cell layer

of the epithelium, the *vascular papillae* and the *lamina propria*. Representative images of the ASIC-2 staining, observed in the functional epithelial cells and in nerve fibers in the *lamina propria*. Representative images of δENaC positive staining observed in nerve endings in the *lamina propria* of *vascular papillae*. In each image, arrows point at positive staining. The dotted-square insert illustrates the staining in the *vascular papillae* and *lamina propria* (right column). EP: epithelium, LP: *lamina propria*. Arrows indicate positive staining. The dotted line delineates the epithelium. The scale of the IFA images is 50 μm.

ASIC-2 staining was localized on the plasma membrane of the functional layer of the esophageal epithelium by both immunohistochemistry and immunofluorescence staining (Figure 4). Moreover, ASIC-2 immunoreactivity showed a fine, dashed line that was clearly visible, very close to the basal layer of the epithelium, including the *vascular papillae* (Figure 4), likely to be ASIC-2-positive sensory nerve fibers. ASIC-2-positive sensory nerves were found in a distribution similar to that of ASIC1. In contrast to ASIC-1, ASIC-2 immunoreactivity was found in the smooth muscle cells of the *muscularis mucosae* by both IHC and IF staining (Figure S4). In the absence of the primary antibody (ASIC-2), no immunoreactivity was found.

The immunofluorescence technique showed δENaC-positive nerve fibers in the *lamina propria*, very close to the basal cell layer of the epithelium (Figure 4). In the absence of the primary antibody (δENaC), no immunoreactivity was found.

4. Discussion

This study describes the main immune cell phenotypes and the presence of acid-sensing receptors in the adult healthy human esophagus. To our knowledge, this is the first time that these immunocytes are assessed and quantified in all tissue layers within the proximal, middle and distal regions of this organ, in the absence of pathology. This characterization may contribute to better understanding esophageal immunity in health and disease and, potentially, establishing reference values of immune cell populations in the human esophagus.

Within this study, immune cells have been identified in all esophageal layers, with the *vascular papillae* and the *lamina propria* being the most infiltrated layers, which could be easily explained by their high degree of vascularization [19]. Similar to other locations of the digestive tract, T lymphocytes are the most abundant immune cells residing in the outer layers of the esophagus, contributing to the protection and maintenance of tissue homeostasis. In this study, we only quantified the two major subtypes characterized by the surface antigens CD4 (helper T cells) or CD8 (cytotoxic T cells), without analyzing the effector or memory phenotype. In previous studies, the identification of specific intraepithelial lymphocytes such as regulatory T cells or Th17, mainly within the inflamed esophagus [20,21], further supports the need for the deep characterization of T lymphocytes in the human esophagus. In fact, in the epithelial compartment, the increase in T lymphocytes features inflammatory esophageal diseases, mainly LE, EoE and GERD [5,22–24], with differential T cell distribution and subtypes, as well as distinctive infiltration of other immunocytes, such as eosinophils and mast cells.

In the small intestine and the colon, B lymphocytes do not infiltrate the epithelium but reside within aggregated and isolated lymphoid follicles and cryptopatches and are also found scattered throughout the *lamina propria*. In our study, we found B lymphocytes rarely infiltrating the healthy esophageal epithelium, absent in both the *muscularis mucosae* and the *muscularis externa*, but present, although not abundant, in the *vascular papillae* and the *lamina propria*. In contrast to B cells, we found that T lymphocytes, both helper and cytotoxic subtypes, are present across all the esophageal layers, especially as intraepithelial lymphocytes, but are even more abundant in the *vascular papillae* and the *lamina propria*. Geboes et al. [25] already described a similar distribution in the healthy esophagus, with scattered intraepithelial T cytotoxic lymphocytes with a protective function, while T helper and B lymphocytes were commonly located in the *lamina propria*. The physiological role

of T lymphocytes in muscular layers is unknown, although they may play a role in motor esophageal disorders, potentially, through the release of toxic mediators. Particularly, in achalasia, an increase in T lymphocytes has been identified in the smooth muscle layers near the myenteric plexus. Of interest for understanding its pathophysiology, the main subpopulation is the $CD8^+$ lymphocyte subtype, which expresses the TIA-1 marker, indicative of active cell degranulation [26]. Moreover, in achalasia, increased infiltration of B lymphocytes [26] and mast cells in the muscle has been described, being associated with the loss of interstitial cells of Cajal and with neuronal degeneration [27]. Despite the fact that the specific role of inflammatory cells is not fully described, an immune-mediated cytotoxic mechanism seems to impair muscular contraction in this disorder.

In non-pathological conditions, we observed that mast cells are abundant in the *vascular papillae* and the *lamina propria,* and only a few of them have an intraepithelial location. Mast cells are commonly known for their role in allergy, yet they are involved in many other protective roles, such as wound healing, intestinal barrier function regulation, angiogenesis, and defense against pathogens [28]. It is believed that mast cells rarely reside within the smooth muscular layer [29,30]; nonetheless, in the present study, we described, for the first time, that mast cells are the most abundant immune cell type in the *muscularis mucosae* and the *muscularis externa,* reaching up to 100 cells/mm^2 in some individuals. The underestimation of the mast cell abundance in the *muscularis externa* can be explained by the use of less specific staining, such as toluidine blue for its quantification, by the low number of healthy subjects included and the difficulty in obtaining transmural specimens [30]. Despite its presence in the *muscularis externa* of the esophagus, only a few studies have assessed the role of mast cells in controlling smooth muscle physiology. Paterson et al. [31] showed that esophageal luminal acid activates neuronal pathways involving mast cell degranulation and the release of excitatory neurotransmitters (substance P and neurokinins), which evokes sustained contraction of the longitudinal smooth muscle layer of the esophagus. Nevertheless, the exact location of these activated mast cells was not determined. To further support the role of mast cells in muscular activity, it has recently been hypothesized that the activation and degranulation of the mast cells present in the *muscularis externa* can affect the esophageal smooth muscle, potentially contributing to motor dysfunction in EoE and achalasia [32].

We corroborate previous findings of small numbers of intraepithelial mast cells in the healthy esophagus [33]. Intraepithelial mast cell numbers were significantly increased in children with erosive esophagitis and further increased in patients with EoE when compared with controls [34]. In contrast, intraepithelial mast cells were not significantly increased in adults with erosive and non-erosive GERD compared with controls [33].

Together with mast cells, macrophages are the most abundant immune cells in the *muscularis mucosae* and the *muscularis externa.* The physiological meaning of the presence of esophageal macrophages in the muscle layers is unknown. Their presence may suggest a possible implication in regulating esophageal motility, as shown in the intestine in both health and disease [35,36], yet experiments to confirm similar findings in the esophagus are lacking. We consistently found the presence of sparse intraepithelial macrophages along the entire esophagus. This novel finding is in contradiction with some studies, in which the authors could not identify intraepithelial macrophages in healthy tissue by targeting the same pan-macrophage surface marker CD68 [33,37]. It is established that macrophages reside in the *vascular papillae* and *lamina propria* of the esophagus of healthy subjects, and that their presence remains unaltered in patients with erosive and non-erosive GERD [33,37]. Despite the fact that *lamina propria* macrophages in the small intestine are responsible for patrolling and eliminating potential pathogens and have been involved in esophageal diseases such as esophageal xanthoma and adenocarcinoma [38,39], their specific role in the esophagus is still understudied.

In contrast to the other segments of the gastrointestinal tract and other types of granulocytes, eosinophils were not observed in any layer of the adult healthy esophagus, in accordance with previous observations in transmural samples obtained from children [40].

As far as is known, the reason for this striking absence is undetermined. The confirmation of these findings in healthy subjects would lead to the conclusion that the presence of even small numbers of eosinophils in any layer of the esophagus would be a histological marker of non-specific disease. The difficulty would be to achieve a proper diagnosis, since the infiltration of intraepithelial eosinophils is described not only in EoE but also in all GERD spectra and in non-esophageal-related diseases, such as respiratory tract allergies, Crohn's disease or gastrointestinal eosinophilia [41–43]. The pathologic diagnosis of EoE can be difficult in some patients due to the overlap with GERD [44] and the variability of eosinophil infiltration along the esophagus, leading to possible underdiagnoses when the tissue is sampled insufficiently [45].

Traditionally, TRPV1 receptors located in sensory nerve endings have been suggested to sense esophageal acid reflux [46], but other receptors and channels can potentially play a role. In the present study, we identified the distribution of the acid-sensing ion channels ASIC-1, ASIC-2, and the δ subunit of the ENaC in the different layers of the distal esophagus. A better knowledge of the expression and distribution of the acid-sensing receptors and acid-induced symptoms in the esophagus is essential for facilitating the development of new therapies for GERD patients. Notably, we found that the acid-sensing receptors ASIC-1, ASIC-2 and δENaC are expressed at the protein level and widely distributed through the inner layers of the distal esophagus, such as the epithelium, *vascular papillae* and *lamina propria*. Our findings are in agreement with Yamamura et al., who showed the mRNA expression of the proton-sensitive δENaC in the esophageal mucosa of the human esophagus by using reverse transcription–polymerase chain reaction and in situ hybridization methods [47]. Moreover, the rat esophagus also contains the acid-sensing receptors ASIC-1, ASIC-2 and ASIC-3 in the epithelium, and the ASIC-3 subtype is present only in the *muscularis mucosae* [48]. The morphology of the staining in the *vascular papillae* and *lamina propria* strongly suggests that these acid-sensing receptors are also expressed in afferent sensory nerve endings, as we already described for the TRPV1 and TRPA1 in the human oropharynx and larynx [49]. Whether these afferent nerves are vagal or spinal needs to be further elucidated, although previous evidence in rodents suggests that esophageal perception is mediated by the vagus nerve [50]. Moreover, Dusenkova et al. already described that most of the esophageal TRPV1-positive vagal afferent neurons also express ASIC-1 and ASIC-2 in the guinea pig [51].

The potential upregulation of these acid-sensing receptors in patients with esophageal hypersensitivity present in GERD and EoE needs to be addressed, as it may play a role in symptom generation during disease [52]. How mast cells and lymphocytes contribute to the esophageal barrier function and the induction of visceral hypersensitivity has been understudied. Studies in rodents show that intraluminal acid impairs the esophageal barrier function and sensitizes esophageal vagal nociceptive C-fibers involving mast cell activation [53,54]. Of note, nociceptive C-fibers [53] and dorsal root ganglion (DRG) neurons express ASIC1, ASIC2 and ASIC3. Interestingly, ASIC1 and ASIC3 are upregulated in patients with reflux esophagitis and in rodent models of esophagitis and non-erosive reflux disease [55]. Even more interesting is that in patients with LA grade-A esophagitis, ASIC1 and 3 expressions are associated with symptoms of heartburn, while the expression of ASIC3 shows a positive correlation with regurgitation [55]. An altered epithelial barrier and/or the upregulation of ASIC receptors may lead to visceral hypersensitivity and symptom perception in GERD.

The limited literature regarding the esophageal deepest layers, given the sampling difficulty, enhances the value of this study, where the main immune cell populations are thoroughly described in all tissue layers, excluding the submucosa. Nonetheless, we acknowledge in this study two main limitations: the small sample size analyzed and the relatively large variability. Evidence suggests that the variability observed for some immune cell populations is somehow intrinsic to the esophageal tissue, since this is also observed in other studies using immunohistochemistry [56,57] and multicolor flow cytometric analysis in healthy tissue [58]. Of note, the findings of the present study refer

to a population aged 57 ± 11 years-old and, due to the influence of age on the immune system, we cannot speculate that a similar distribution will appear in younger and older subjects until additional studies are performed. We could expect that the distribution of cell populations and sensing receptors has age-dependent physiological consequences on the esophagus, such as defensive activity or disease predisposition. In fact, age is a risk factor in developing esophageal dysfunction and, in disease, age can determine a differential phenotype. One example is EoE, which is characterized by an inflammatory phenotype in pediatric patients, while a fibrostenotic phenotype is more frequent in adult patients [59,60].

In conclusion, the present study offers a basal characterization of the distribution of immunocytes in the adult esophagus, unveiling novel directions for the study of esophageal diseases, such as motility disorders. The high number of macrophages and mast cells within the muscular layers may underlie physiological muscle activity and, potentially, may play a role in clinical manifestation and symptom severity. We also show that the distal esophagus expresses acid-sensing receptors such as ASIC-1, ASIC-2 and the δ subunit of the ENaC, other than the well-described TRPV-1 receptor, which could play a role in esophageal hypersensitivity. Additional studies are needed to validate the findings of our study, including larger cohorts with broader age range, to also interpret if sex and age influence cell distribution and predispose to esophageal disorders. Future research is warranted to better understand the contribution of esophageal immunity and acid-sensing receptors to esophageal homeostasis and dysfunction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/gastroent15030058/s1, Figure S1: Representative images of immunostainings for each immune cell population in the esophageal epithelium. Bottom row images correspond to the magnification of the squared box in the top row (magnification top row 400×); Figure S2: Positive control staining. Human healthy duodenal or jejunal sections were processed as routine tissue for control staining of the antibodies assayed in the study. Representative images are shown for each immune cell population (magnification 400×); Figure S3: Immune cells distribution in esophageal layers in the three regions analyzed. Kruskall Wallis test was performed for every cell type comparison. EP: epithelium, VP: vascular papillae; LP: lamina propria, MM: muscularis mucosae, ME: muscularis externa Dunn's multiple comparison test was used for adjusted *p*-value calculation (* p = 0 .05; ** p = 0.01; *** p = 0.001; **** $p < 0.0001$); Figure S4: ASIC-2 immunoreactivity in the muscularis mucosa of human esophagus visualized by IHC (left) and IF (right). Pictures correspond to two different specimens. Scale is 50 μm; Table S1: Primary antibodies and experimental conditions for the immunohistochemical procedures; Table S2: Secondary antibodies. Primary antibodies used, with product number company information and dilution factor.

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Informed Consent Statement: No informed consent was obtained from the donors. Belgian legal aspects concerning the research on tissue from a death organ donor (Wet 7mei2004) were followed, and ethical approval was granted by the local Ethical Committee (University of Leuven; S56978-ML10868). Spanish legal aspects (Llei 30/1979 y RD 1723/2012) were followed, and ethical approval was granted by the Hospital Vall d'Hebron [PR(AG)94/2013].

Data Availability Statement: All data generated or analyzed during this study are included in this article. Data obtained in this study are not available on a public repository but are available upon editorial/reviewer's request. Further enquiries can be directed to the corresponding author.

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Abbreviations

GERD (gastroestophageal reflux disease); EoE (eosinophilic esophagitis); LE (lymphocytic esophagitis); Th (T helper lymphocytes); Tc (T cytotoxic lymphocytes); EP (epithelium); VP (*vascular papillae*); LP (*lamina propria*); MM (*muscularis mucosae*); ME (*muscularis externa*).

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