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ORIGINAL ARTICLE **Clinical Mechanisms in Allergic Disease**

# Dietary treatment modulates mast cell phenotype, density, and activity in adult eosinophilic oesophagitis

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**Summary**

*Background* Mast cells (MCs) are abundant in the inflammatory infiltrate in eosinophilic oesophagitis (EoE), but decrease with disease remission. However, their phenotype, role in the pathophysiology of the disease, and modulation after effective dietary therapy are still unclear.

*Objective* To define the phenotype of oesophageal MCs, their modulation through dietary therapy, and their association with clinical manifestations of EoE.

*Methods* Oesophageal mucosal samples from 10 adult patients with EoE obtained before and after effective six-food elimination diet (SFED) therapy, as well as from 10 control subjects were analysed. Eosinophil and MC density were quantified. Gene expression of chemoattractants for eosinophils (CCL11, CCL24, and CCL26), MCs (SCF), and their receptors (CCR3 and SCFR, respectively) were assessed by means of qPCR. Gene and protein expression of specific MC proteases (CPA3, CMA, and TPSB2) were evaluated with qPCR and immunofluorescence. Clinical manifestations and atopic background were recorded.

*Results* MC density was significantly increased in EoE compared with controls, decreasing after dietary treatment (18.6 to 1.44 cells/hpf, respectively; $P < 0.001$). The $MC_{TC}$ subtype predominated in the oesophageal mucosa (90%) in both patients with EoE and controls. Gene expression of MC-related proteases, eotaxins, and SCF were up-regulated in patients with EoE, but significantly decreased after therapy, regardless of atopic background. Epithelial peaks of MCs and eosinophils were significantly associated ($\rho = 0.80$) in EoE and correlated with the symptom score ($\rho = 0.78$). Gene expression of MC proteases and eotaxins also correlated with the symptom score ($P < 0.05$).

*Conclusions and Clinical Relevance* MC and its proteases seem to play a relevant role in the pathophysiology and symptoms of EoE, which can be reversed after effective dietary treatment.

**Keywords** carboxypeptidase A3, CCL24, CCL26, CCR3, chemokines CCL21, chymase, dietary treatment, Eosinophilic oesophagitis, mast cells, SCF, SCFR, six-food elimination diet, tryptase

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## Introduction

Eosinophilic oesophagitis (EoE) is a chronic food-triggered, immune-mediated disease of the oesophagus. Clinically, EoE is characterized by symptoms of oesophageal dysfunction, while histologically, it is marked by an inflammatory infiltrate with large numbers of both intraepithelial eosinophils and mast cells in the oesophageal epithelium [1]. In the past few years, EoE has rapidly risen in both incidence and prevalence [2–4] so

that it is now the most likely cause of dysphagia among young patients.

A role for mast cells in the pathogenesis of the disease has been proposed [5–8] after studies demonstrated both their activation [8] and increased density in the oesophageal mucosa of experimental [9, 10] and human EoE in adults [11–14] and children [8, 15–19]. These increases were significant compared with healthy controls as well as with patients with gastro-oesophageal reflux disease (GERD); in fact, mast cell density has been proposed as a marker to distinguish GERD from EoE [15, 20].

The potential role played by mast cells in EoE is supported by several pieces of evidence, most of it indirect. For example, the density of mast cells correlates with eosinophilic infiltration within the oesophageal epithelium [21], with a reduction in both cell types after treatment with topical steroids [22–24] or anti-interleukin-5 [25] and in association with clinical remission [12, 24, 26]. The expression of specific mast cell-mediators has also been shown to be up-regulated in several reports [8, 16, 18], with mast cell-derived TGF-β1 contributing to oesophageal dysmotility in both human [18] and experimental (murine) EoE [9] through the induction of smooth muscle hypertrophy and hyperplasia. Previous research supports the role of these cells in local IgE-mediated reactions against certain allergens, as IgE production and $IgE^+$ mast cells are present in the oesophageal epithelium of these patients [13, 19]. However, their contribution to the aetiopathogenesis of EoE remains unclear.

Mast cells are mesenchymal bone marrow-derived myeloid cells that are widely distributed in vascular connective tissue as a part of the innate immunity elements against parasites and bacteria. Human mast cells are classified into two types depending on their granule content [11, 27]: $MC_T$ (mast cells with tryptase) and $MC_{TC}$ (mast cells with tryptase and chymase). Typically, $MC_T$ are located in the mucosal tissue while $MC_{TC}$ are found mainly in connective tissues, but they can also be found in the submucosa and, rarely, in the muscularis propria of the digestive tract [28–30]. This phenotypic diversity is not only a descriptor of tissue location [31], but also of the regulation of cytokine gene expression and, as such, is associated with functional differences [32].

In recent years, dietary therapies have emerged as a drug-free treatment alternative for inducing and maintaining disease remission in both paediatric and adult patients with EoE [33]. According to a recent systematic review [34], an empiric six-food elimination diet (SFED) is currently the best dietary approach for inducing histological remission of EoE. The anti-inflammatory properties of SFED are exerted by removing antigenic luminal stimuli from the diet of sensitized patients [35–38], allowing the recovery of oesophageal tissues without inducing apoptosis in inflammatory cells or modifying signalling pathways, which commonly occurs when steroids, immunomodulators, or biological therapies are used. Despite mast cells being the main effector cells in IgE-associated responses and playing a central role in allergic responses [39], to date, the ability of dietary therapies to reduce mast cell density and/or activity has not been fully elucidated.

The aims of this study were to analyse the phenotype of oesophageal mast cells and the effect of an SFED on the eosinophil and mast cell infiltrate in EoE. The contribution of mast cell activity to clinical remission will also be studied to gain further insight into the aetiopathogenic mechanisms of this disease.

## Material and methods

### *Study design*

A controlled, quasi-experimental design was used. Patients with EoE and control subjects were recruited, and clinical symptoms were recorded. Oesophageal biopsies were obtained from each participant at baseline and, in patients with EoE, after 6 weeks of an empiric SFED. Biological assessment of tissue samples and clinical evolution were analysed to evaluate the response to dietary treatment.

### *Participants and clinical assessment*

Adult patients with EoE who were naïve to topical or systemic steroid therapy for EoE were prospectively recruited from October 2011 through March 2012. Diagnosis for EoE was based on widely accepted criteria [1] which included (i) infiltration of oesophageal epithelium by 15 or more eosinophil leucocytes per high-powered field (hpf); (ii) absence of eosinophilic infiltration in biopsy specimens from gastric and duodenal mucosa; (iii) ruling out of proton pump inhibitor-responsive oesophageal eosinophilia as defined by the persistence of eosinophilic infiltration after an 8-week course of omeprazole (20 mg/twice a day); and (iv) ruling out drug intake, parasites, oesophageal causticications, haematologic neoplasms, or other events in the patient's medical history as possible causes of oesophageal eosinophilia.

Gender-matched control samples were obtained endoscopically from individuals who had been consecutively referred to undergo endoscopy under sedation during the study period due to symptoms of dyspepsia or a suspected gastroduodenal ulcer. All selected control subjects exhibited a normal endoscopic appearance of the oesophagus; hiatal hernia, incompetent cardias, and oesophageal peptic lesions were excluded, and the

analyses of oesophageal mucosal biopsies were also reported as normal. Wherever possible, clinical histories of all participants were used to assess family and/or personal background of atopy (Table 1; see also Table S1).

Oesophageal symptoms were assessed structurally by means of a score validated for achalasia [40], but previously used in adult EoE [37, 41]. The duration and intensity of the dysphagia events along with the frequency and intensity of heartburn and regurgitation were recorded both at the beginning of the study and after dietary treatment.

### *Endoscopy and biopsy sampling procedure*

All endoscopic exams were carried out under conscious sedation by a board-certified gastroenterologist (AJL); they were performed with a flexible 9-mm-calibre Pentax EG-2770K gastroscope (Pentax of America, Inc, Montvale, NJ, USA) with a 2.8-mm work channel. The calibre and appearance of the oesophageal wall were recorded for all participants during the endoscopic procedure. Biopsies were taken with the aid of a standard needle biopsy forceps (Endo Jaw FB-220U, Olympus Medical Systems, Tokyo, Japan) from both the upper and lower oesophageal thirds; a minimum of five specimens were obtained from each location. These were then fixed in 4% formalin and routinely processed for histopathological analysis. Three additional endoscopic samples from the middle oesophageal third of all study subjects were collected during the same endoscopic procedure and preserved in an RNA stabilization solution (RNAlater; Ambion, Inc, Austin, TX, USA) at −80 °C until being processed for gene expression studies. No specific complications were observed in any patient after the biopsy procedure.

### *Treatment and follow-up period*

All patients diagnosed with EoE were asked to follow an SFED for a 6-week period, avoiding the consumption of six-food groups reported to cause food allergies, namely cereals, milk and dairy products, eggs, fish/seafood, soya/legumes, and nuts [37]. The patients were given an amino acid-based formula adapted to oral consumption (Neocate Advance, 100 g sachets, banana & vanilla flavours, SHS International, Liverpool, UK) in order to supplement their diets. Written information about which foods should be avoided and which allowed, along with instructions to read food labels carefully, were provided to patients by board-certified gastroenterologists in our department. A telephone number and e-mail address were also provided to patients in case of further doubts regarding the SFED. Only oesophageal samples from patients who showed diet-induced remission of EoE were considered for comparative analysis.

### *Histological study*

Oesophageal mucosal samples were fixed in formalin, embedded in paraffin, and routinely processed for haematoxylin and eosin staining. The histological analysis was performed by an experienced pathologist (JLY-C) blinded to the experimental groups. The peak number of eosinophils was counted in the most densely inflamed areas with the aid of Nikon Eclipse 50i (Nikon Corp, Tokyo, Japan) light microscopy in 3 high-power

**Table 1.** Clinical characteristics of patients with EoE included in the study

| Patients | Age (years) | Sex | Time of evolution (months) | Symptoms | Endoscopy | | Family background of atopy | Personal background of atopy | Identified trigger food |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Calibre | Mucosal appearance | | | |
| 1 | 25 | M | 12 | FI, Dy | N | LF, Rg | No | No | F&S &Ri |
| 2 | 18 | M | 60 | FI, WL | N | LF, C | Sister: D | AR | Le, Nu&Ri |
| 3 | 38 | M | 4 | Dy, AP | R | WP, Rg | No | BA, AR | Mi, Eg, Ri, F&S, Le & So |
| 4 | 36 | M | 36 | FI | N | LF, WP, Rg | Brother: FS | BA, AR | Mi, Ri, Nu& So |
| 5 | 38 | F | 60 | FI, Dy | N | LF, WP | Sister: AR | BA, AR | Leg&Nu |
| 6 | 18 | M | 24 | AP, V | N | LF, Rg | No | AR, FS | Mi, F&S, Le, Nu&Ri |
| 7 | 51 | F | 24 | FI, Dy | N | LF, WP, Rg | No | No | Mi & Le |
| 8 | 34 | M | 48 | FI, Dy, Ht | R | LF, WP, C, Rg | Father: BA; Brother: AR | No | Mi |
| 9 | 38 | M | 120 | FI, Dy | N | Normal | No | BA, AR, FS | Ri |
| 10 | 35 | M | 120 | Dy, AP | N | Rg, C | Brother: DS | BA, AR, FS | Mi, F&S &Ri |

Sex: M, male; F, female. Symptoms: FI, food impaction, Dy, dysphagia, AP, abdominal pain, V, vomiting, Ht, heartburn, WL, weight loss. Endoscopy: N, normal; R, reduced; Rg, rings; LF, longitudinal furrows; C, crêpe-paper appearance; WP, white plaques. Atopy: BA, bronchial asthma; AR, allergic rhinitis; FS, food sensitivity; D, dermatitis; DS, drug sensitivity. Food triggers: Mi: milk; Ri: rice; F&S: fish & seafood; Le: legumes; Nu: nuts; Wh: wheat; Co: corn; Eg: eggs; So: soya.

fields (0.212 $mm^2$). Peak eosinophil count per hpf was calculated in the epithelial strata by averaging the eosinophil counts.

*Immunofluorescence*

Formalin-fixed, paraffin-embedded tissues were sectioned at 5 μm. Cuts were first deparaffinized and rehydrated following standard procedures and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. After treatment with blocking solution (DakoDiagnósticos, Barcelona, Spain) for 60 min at room temperature, samples were simultaneously incubated overnight at 4 °C either with the primary antibodies antitryptase (TPSB2, Dako) and antichymase (CMA, Abcam, Barcelona, Spain) or with antitryptase and anticarboxypeptidase (CPA Abcam). Samples then underwent a subsequent 30-min incubation at room temperature with the secondary antibodies Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Madrid, Spain). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The negative control slides were made in the same fashion except no primary antibodies were added. Fading was controlled using the Prolong antifade mounting medium (Molecular Probes). Positive cells in the epithelium, the papillae, and the lamina propria were counted with the aid of a fluorescence microscope (BX61, Olympus, Barcelona, Spain) at high magnification (400×) in 10–12 non-overlapping fields. Results are expressed as the number of positive cells/hpf in each anatomical location, as well as the percentage of $CMA^+$ or $CPA^+$ cells with respect to the $TPSB2^+$population.

*Analysis of RNA expression*

Total RNA was isolated with the Mir*Vana*™ miRNA Isolation Kit (Ambion), following the manufacturer's instructions. Gene expression for the chemotactic factors for eosinophils (CCL11, CCL24, and CCL26), mast cells (SCF and TGF-β), and their receptors (CCR3 and SCFR, respectively), along with mast cell-specific proteases (CPA3, CMA, and TPSB2) were evaluated in all samples. Each assay and its assay ID number are available at Applied Biosystems (Madrid, Spain) (see Table S2). Simultaneous real-time PCRs were performed with TaqMan Low-Density Arrays (Applied Biosystems) preconfigured in a 384-well format and spotted on a microfluidic card. Each TaqMan Gene Expression Assay consists of a forward and reverse primer at a final concentration of 900 nM and a TaqMan MGB probe (6-FAM dye-labelled; Applied Biosystems), with a final concentration of 250 nM. The assays are gene specific and have been designed to span an exon–exon junction. Thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). This procedure was replicated twice for each gene and each sample, with water as a negative control.

Relative changes in mRNA expression were calculated with the cycle threshold (Ct) method [42] with the aid of Sequence Detection System 2.1 software (Applied Biosystems). Expression levels of target genes were normalized to 18S, GAPDH, PGK1, GUSB, and β-actin expression.

The amount of mRNA for each gene was calculated in each sample using the Ct value. Relative gene expression was calculated as follows: $2\Delta\Delta Ct$, where $\Delta\Delta Ct = \Delta Ct$target gene $- \Delta ct$ control genes. The fold change for the treatment was defined as the relative expression compared with the corresponding control and was calculated as follows: $2\Delta\Delta Ct$, where $\Delta\Delta Ct = \Delta Ct$patient $- \Delta Ct$healthy.

*Statistical analysis*

We calculated the optimal sample size based on our previous results [37], from which we observed that patients with EoE had a mean eosinophil count of 47.9 (25.6) eos/hpf and that after dietary treatment, the number of eosinophils decreased significantly to 3.5 (3.9) eos/hpf. Drawing on these results and aiming for a power of 90%, five individuals would be needed to observe these differences. In the end, 10 patients were selected to detect possible differences in both mast cells and gene expression.

Means and standard deviations were reported for continuous variables and are expressed as 'mean (standard deviation)' throughout the text. Proportions were reported for categorical data. Results are expressed as a median with an interquartile rank (IQR) for scoring clinical symptoms. Comparisons between groups (control subjects and patients with EoE) were performed with nonparametric tests: the Mann–Whitney *U*-test for quantitative variables and the Fisher's exact test for nominal variables. For comparison before and after SFED treatment, the nonparametric-paired Wilcoxon signed-rank test was used. Nonparametric correlations (Spearman's rho) were used for relationships between eosinophils, mast cells, gene expression, and clinical symptoms. A 0.05 level of significance was used throughout. Statistical analyses were performed with the aid of PASW 18.0 statistical analysis software (SPSS Inc, Chicago, Ill).

### Ethics

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board of our hospital. Informed consent was obtained from all patients prior to all endoscopic exams.

## Results

### Study population

A total of 10 patients with EoE (eight men) and 10 gender-matched control subjects were included in the analysis. The groups had a mean age of 33.1 (10.1) and 53 (19.9) years, respectively. Individual clinical characteristics of the experimental subjects are given in Table 1 and Table S2. Mean duration of symptoms in patients with EoE exceeded 4 years (50.8 ± 40.9 months), with dysphagia and food impaction being the most common, exhibited by 70% of patients. No difference in clinical manifestations was observed between atopic and non-atopic subjects (Table 2).

### Eosinophils and mast cell density, chemoattractants, and the effects of dietary treatment

In the EoE group, peak intraepithelial eosinophil density was 56.8 (29.9) cells/hpf, which decreased to 3 (4.2) cells/hpf after SFED-based treatment ($P < 0.001$). No intraepithelial oesophageal eosinophils were detected in any of the controls. Peak counts for intraepithelial mast cells in patients with EoE were 18.6 (15.2) cells/hpf, much higher than for the control group, which had a peak count of 0.5 (0.6) cells/hpf ($P < 0.001$). As before, after SFED, mast cell density decreased to 1.44 (1.7) cells/hpf ($P < 0.001$) (Figs 1 and 2). No differences between atopic and non-atopic patients with EoE were detected in eosinophil [55 (30.4) and 61 (34.8) cells/hpf, respectively] or mast cell counts [20 (18.1) and 15.3 (5.2) cells/hpf, respectively].

Active eosinophil recruitment was demonstrated by identifying overexpression of all the eotaxins in the EoE group in comparison with the controls: CCL11 (8.5-fold increase), CCL24 (12.2-fold increase), and CCL26 (51.1-fold increase, $P < 0.05$ for all), which is in good agreement with previous studies [21, 43]. Dietary treatment significantly decreased eosinophil infiltration and all eotaxin expression to control group values

**Table 2.** Clinical characteristics and gene expression levels of atopic and non-atopic patients with EoE

| | | Atopic *vs.* Non-atopic | *P* |
|---|---|---|---|
| Time of evolution (months) | | 60.6 (45.1) *vs.* 28 (18.3) | 0.250† |
| Symptom Score | | 9 (5.9) *vs.* 6 (2) | 0.723† |
| Symptoms | Dysphagia | 57.1% *vs.* 100% | 0.475* |
| | Food impaction | 57.1% *vs.* 100% | 0.475* |
| | Abdominal pain | 42.9% *vs.* 0% | 0.475* |
| | Heartburn | 0% *vs.* 33.3% | 0.300* |
| | Vomiting | 14.3% *vs.* 0% | > 0.999* |
| | Weight loss | 14.3% *vs.* 0% | > 0.999* |
| Endoscopy Findings | Reduced calibre | 14.3% *vs.* 33.3% | > 0.999* |
| | Normal mucosa | 14.3% *vs.* 0% | > 0.999* |
| | Longitudinal furrows | 57.1% *vs.* 100% | 0.475* |
| | Rings | 71.4% *vs.* 66.7% | > 0.999* |
| | Crêpe-paper appearance | 28.6% *vs.* 33.3% | > 0.999* |
| | White plaques | 42.9% *vs.* 66.7% | > 0.999* |
| Peak eosinophil count | | 55 (30.4) *vs.*61 (34.8) | 0.908† |
| CCL11 gene expression | | 0.41 (0.94) *vs.* 0.15 (0.14) | 0.425† |
| CCL24 gene expression | | 1.5 (2.1) *vs.* 0.91 (1) | 0.732† |
| CCL26 gene expression | | 167 (165.1) *vs.* 275 (327.5) | 0.305† |
| CCR3 gene expression | | 0.01 (0.01) *vs.* 0.08 (0.09) | 0.305† |
| Peak mast cell count | | 17.4 (15.9) *vs.* 15.2 (10.5) | 0.909† |
| TGF-beta gene expression | | 1.1 (0.3) *vs.* 0.89 (0.2) | 0.305† |
| SCF gene expression | | 11.7 (10) *vs.* 13.6 (12.5) | 0.909† |
| SCFR gene expression | | 9.3 (6.6) *vs.* 5.8 (4.4) | 0.210† |
| CPA3 gene expression | | 18.6 (17.3) *vs.* 25.3 (65.1) | 0.732† |
| CMA gene expression | | 2.1 (2) *vs.* 5.9 (6.5) | 0.456† |
| TPSB2 gene expression | | 2.4 (3.9) *vs.* 4.4 (3.9) | 0.909† |

*Chi-square test. †Mann–Whitney *U*-test.

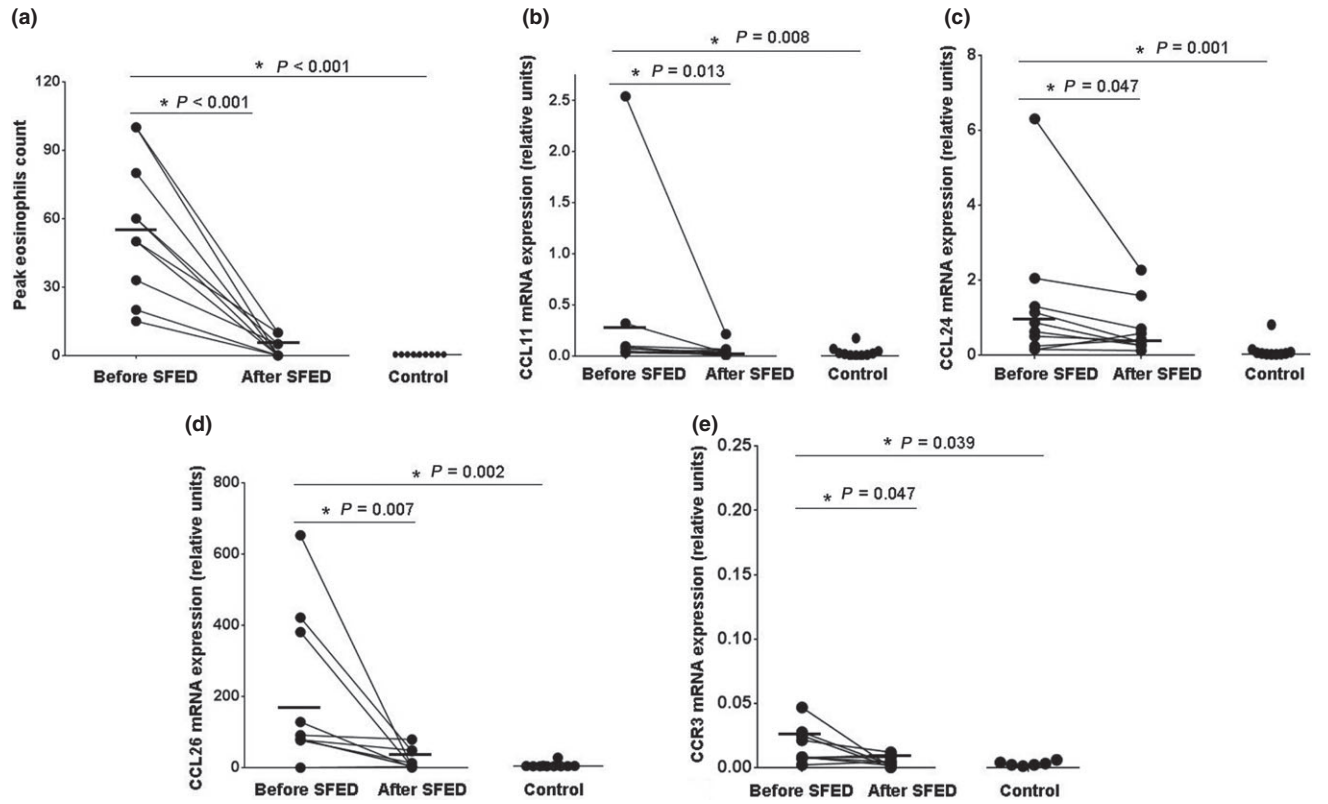


Fig. 1. Eosinophil density and expression of eosinophil chemoattractant molecules. (a) density of intraepithelial eosinophils in patients with EoE before and after effective treatment with a six-food elimination diet (SFED) and in control subjects. Gene expression of eosinophil–chemotactic chemokines eotaxin-1/CCL-11 (b); eotaxin-2/CCL24 (c); and eotaxin-3/CCL-26 (d) in oesophageal mucosal samples from patients with EoE, at baseline and after SFED-induced disease remission, compared with control samples. (e) changes in gene expression of eotaxin receptor CCR-3 in the same samples, at baseline and after an effective SFED. Individual changes in cytokine gene expression are provided. Horizontal bars represent means. *Statistically significant differences ($P < 0.05$) before and after treatment in patients with EoE.

($P < 0.05$). Moreover, the expression of CCR3, the common receptor for eotaxins, was also up-regulated (3.7-fold increase) in patients with EoE, decreasing to control levels after SFED-based treatment (Fig. 1).

In mast cells, chemotaxis was identified through an increase of mRNA in SCF and its receptor (SCFR). In patients with EoE, these values went up 5.6-fold and 3.7-fold, respectively ($P < 0.05$), in comparison with the control group. SFED-based treatment restored SCF gene expression to control values and also reduced SCFR, although not in a statistically significant manner (Fig. 2).

### *Mast cell phenotype and density in EoE and the effects of dietary treatment*

In the control group, 100% of mast cells displayed the MCTC phenotype, although with low density, and 89.3% (±15.6) also contained CPA. In patients with EoE, the proportion of $MC_{TC}$ cells decreased from 100% to 90.2% (±18.8) in the epithelium ($P = 0.020$), a reduction that was reversed after dietary treatment. No significant changes in the mast cell phenotype within the vascular papillae or the lamina propria were observed (data not shown). The number of $CPA^+TPSB2^+$ cells/hpf in the epithelium and the vascular papillae was higher in patients with EoE than in the controls. Dietary treatment reversed this increase in all tissues studied (Fig. 3). The density of $CMA^+TPSB2^+$ cells/hpf in the epithelium of active EoE was also reduced to control values after dietary treatment (Fig. 4).

### *Mast cell activation and modulation through dietary treatment*

Mast cell activity was assessed by quantifying the gene expression of specific mast cell proteases. In EoE samples, all molecules were overexpressed in comparison with control samples: CMA (3.2-fold increase), CPA3 (3.2-fold increase), and TPSB2 (1.7-fold increase, $P < 0.05$ for all); all were reduced to control values ($P < 0.05$) after dietary treatment (Fig. 5). Moreover, no differences in mast cell counts or expression of mast cell-related genes were observed between atopic and non-atopic patients with EoE (Table 2).

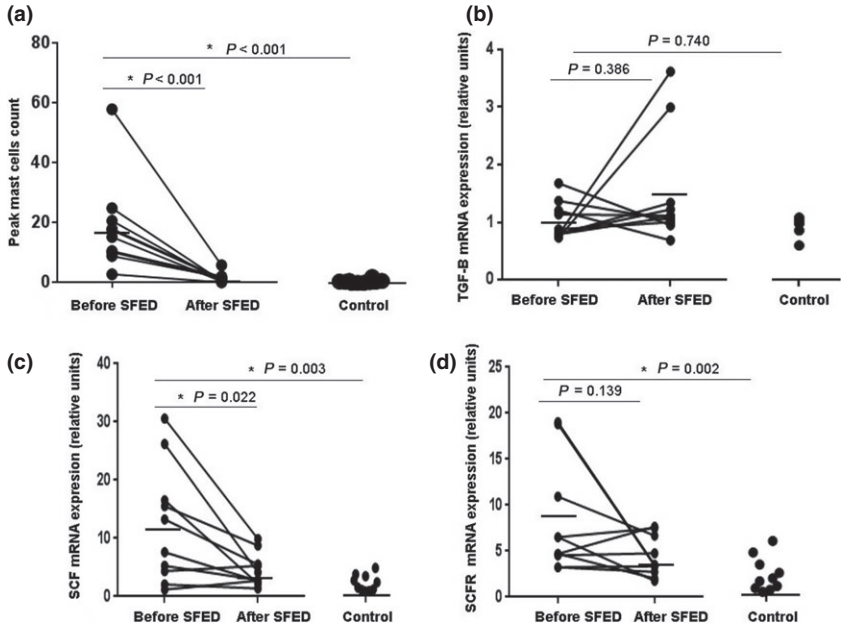


Fig. 2. Mast cell density and expression of mast cell chemoattractant molecules. (a) density of intraepithelial mast cells in patients with EoE before and after six-food elimination diet (SFED)-induced remission and in control subjects. (b) changes in mast cell-derived TGF-β gene expression in the same samples. (c) Gene expression of mast cell chemoattractant stem cell factor (SCF) and its receptor SCFR (d) in oesophageal mucosal samples from patients with EoE, at baseline and after SFED-induced disease remission, and in control samples. Individual changes in cytokine gene expression are provided. Horizontal bars represent means. *Statistically significant differences ($P < 0.05$) before and after treatment in patients with EoE.

### *Modulation of clinical symptoms through dietary treatment*

EoE-associated symptoms were significantly reduced in every patient with EoE after dietary treatment (Fig. 6). Dysphagia (any intensity) was completely resolved in over 70% cases, while food impaction disappeared in 85% of patients. No significant differences in symptom scores in relation to the age or sex of the patients was observed nor did disease duration correlate with the degree of symptom score improvement (data not shown).

### *Relationship between eosinophils, mast cells, gene expression, and clinical symptoms*

The number of eosinophils was significantly correlated to the number of mast cells in EoE oesophageal samples ($r_s$=0.808; $P < 0.001$). The density of both eosinophils and mast cells was strongly associated with the symptom score ($r_s = 0.895$ and $r_s = 0.782$; $P < 0.001$, respectively); likewise, cellular infiltration was also associated with gene expression of major chemotactic factors, including CCL26 ($r_s = 0.706$; $P = 0.001$ with eosinophils), CCL11 ($r_s = 0.452$; $P = 0.045$ with eosinophils), and SCF ($r_s = 0.39$; $P = 0.085$ with mast cells). These correlations were independent of atopic background.

There was also a significant association between the number and activation of mast cells in EoE, as demonstrated by the correlation between mast cell peak and gene expression of CPA3 ($r_s = 0.54$; $P < 0.05$), CMA ($r_s = 0.49$; $P < 0.05$), and TPSB2 ($r_s = 0.49$; $P < 0.05$) proteases. Moreover, mast cell protease expression was associated with oesophageal symptom score (Table 3). There was no association between the number of foods triggering EoE and the number of eosinophils ($P = 0.840$) or mast cells ($P = 0.832$) (Table 3).

## Discussion

Our results demonstrate the effectiveness of dietary treatment in adult EoE, both in reducing mast cell density and activation, and in disease remission, providing proof of the major role these cells play in the pathophysiology of the disease. Moreover, we found that mast cell and eosinophil infiltration in the oesophageal epithelium were directly associated and significantly correlated with clinical symptoms in adult patients with EoE. Additionally, significant relationships between symptoms and the expression of major mast cell proteases were demonstrated as well as with chemoattractant stimuli for both cell types. Finally, to the best of our knowledge, this is the first time that researchers have determined that the mast cell population within the

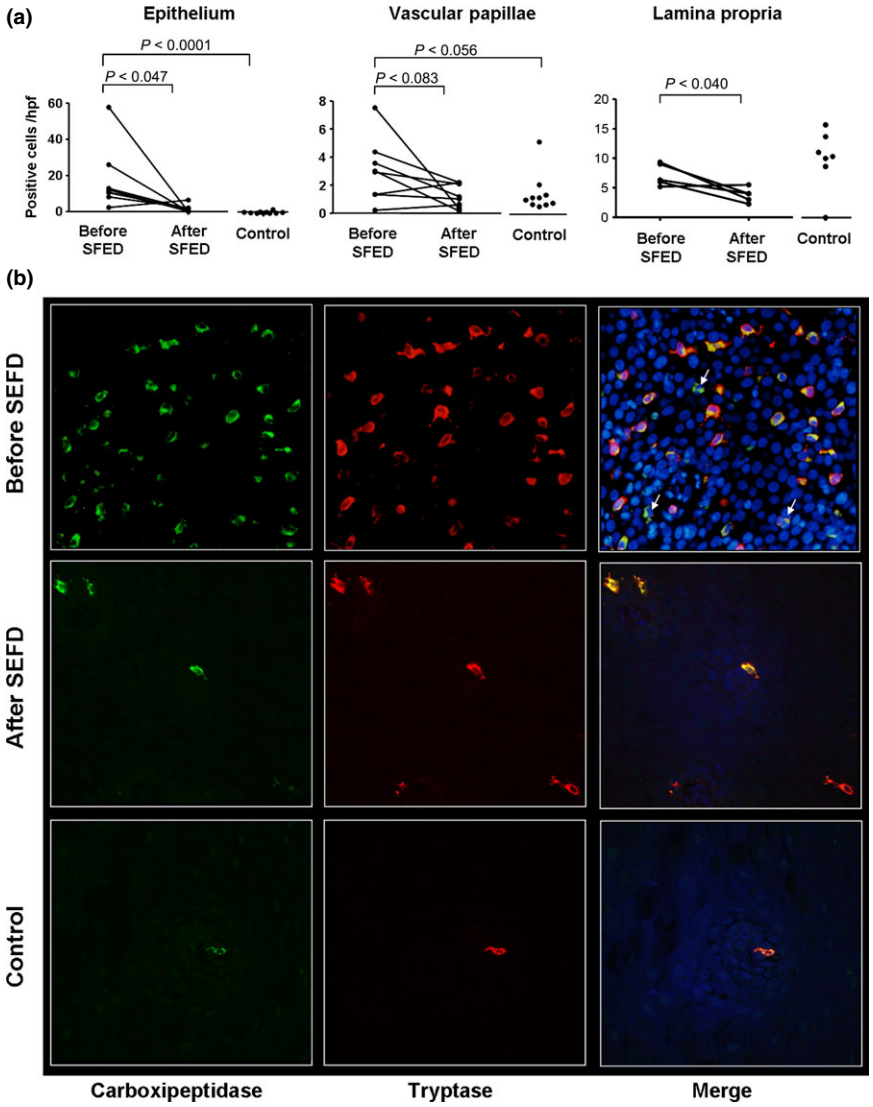


Fig. 3. Histological evaluation of mast cells in the oesophageal mucosa. (a) Individual cell counts per hpf of carboxypeptidase-positive cells in the epithelium, vascular papillae, and lamina propria of patients with EoE before and after dietary treatment, and in the control group. (b) Representative images of the double immunofluorescence for carboxypeptidase and tryptase staining in the three experimental groups. carboxypeptidase-positive mast cells infiltrate the epithelium and the vascular papillae in EoE. Dietary treatment reduced cell density and positive cells were then mainly detected in the vascular papillae. Eosinophils are identified within the epithelium, based on the nuclear morphology (white arrows). Note: SFED: six-food elimination diet.

oesophageal epithelium predominantly consists of $MC_{TC}$ cells, both under normal conditions and in EoE. These cells are also predominant in the skin, nasal mucosa, and intestinal submucosa, but not in the small intestinal mucosa [44]. $MC_{TC}$ cells do not specifically respond to mast cell-stabilizer drugs such as sodium cromoglycate in the same way as $MC_T$ cells, which are predominant in the bronchial mucosa and alveolar wall, a finding which explains the documented lack of efficacy of these drugs in treating EoE [1, 45].

Antigen cross-linking of IgE antibodies on the mast cell surface is the most extensively studied mechanism for the activation and degranulation of these cells. This leads to the rapid release of autacoid mediators and the sustained synthesis and release of cytokines, chemokines, and growth factors [46], which can characteristically lead to anaphylaxis. However, immediate systemic reactions to the foods responsible for EoE are not described in these patients, despite the fact that local IgE production has been demonstrated in the oesophageal mucosa of patients with EoE regardless of their atopic background [19]. Moreover, IgE-bearing mast cells are present in the oesophageal epithelium of patients with EoE exhibiting a personal atopic history [6, 47]. It is worth

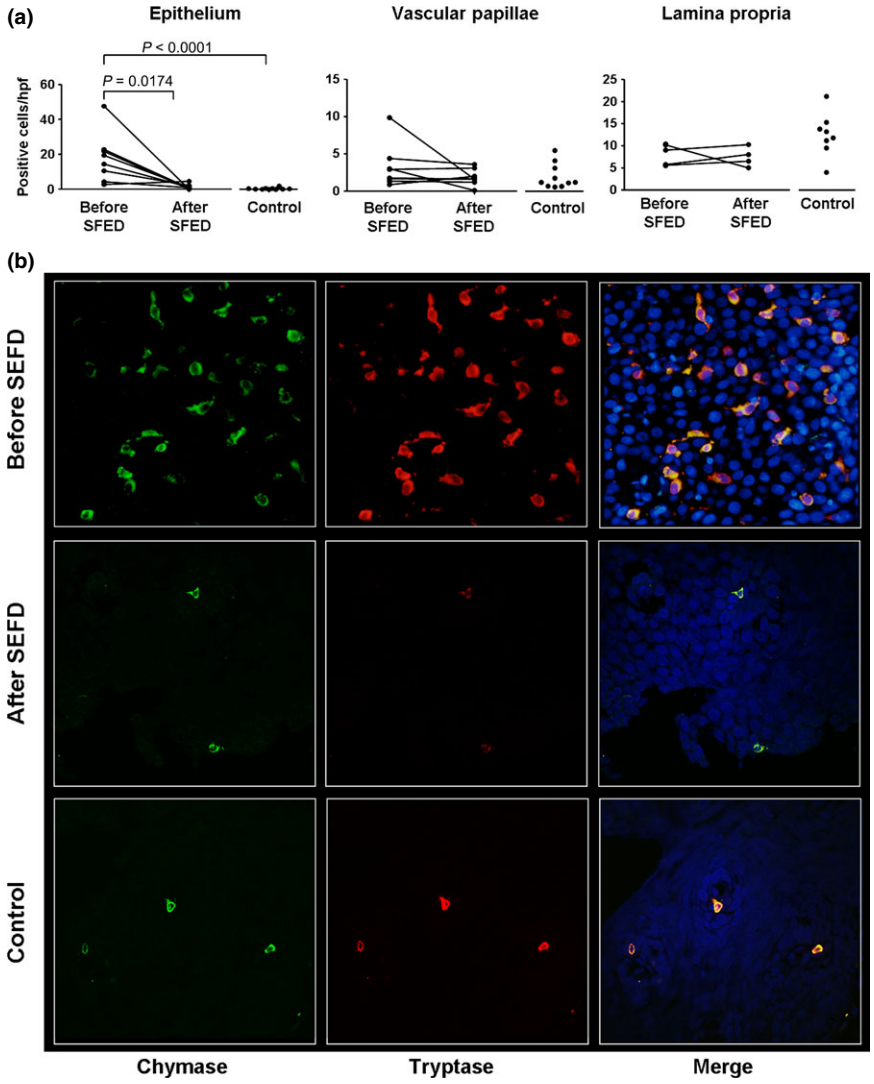


Fig. 4. Histological evaluation of mast cells in the oesophageal mucosa. (a) Individual cell counts per hpf of chymase-positive cells in the epithelium, vascular papillae, and lamina propria of patients with EoE before and after dietary treatment, and in the control group. (b) Representative images of the double immunofluorescence for chymase and tryptase staining in the three experimental groups. Chymase-positive mast cells infiltrate the epithelium in EoE. Dietary treatment reduced cell density, and positive cells were then mainly detected in the vascular papillae. Note: SFED: six-food elimination diet.

noting that three of the 10 patients in our study showed no allergic background, and no differences were noted regarding mast cell counts or activation between atopic and non-atopic patients. This suggests that IgE is not the principal trigger of mast cell activation in EoE. In fact, $MC_{TC}$ are also strong responders to non-IgE-mediated regulatory stimulus including the activation of tolllike receptors [39] or non-immunological mechanisms [48, 49]. The latter include exposure to GER acid [50–52], bile acids [53], or immune mediators, as well as enteric nervous system activation [54]. Among these IgE-independent mechanisms for mast cell activation, one of the most relevant is the ability of certain eosinophil-derived proteins, mainly major basic protein (MBP), to induce mast cell degranulation in an especially attractive, albeit hypothetical, mast cell/eosinophil interaction [55]. In fact, a direct relationship between the density of eosinophils and mast cells has been demonstrated both in our research and in previous reports [21, 25]. Mast cell density (as determined through cell counts in either tryptase, chimase or carboxypeptidase A3-positive cells) directly correlated with oesophageal symptoms in our 10-patient series; we also found a direct association with gene expression levels of the same genes.

Recent advances have provided a plausible explanation for the ability of certain dietary components to initiate and promote EoE, independent of the primary

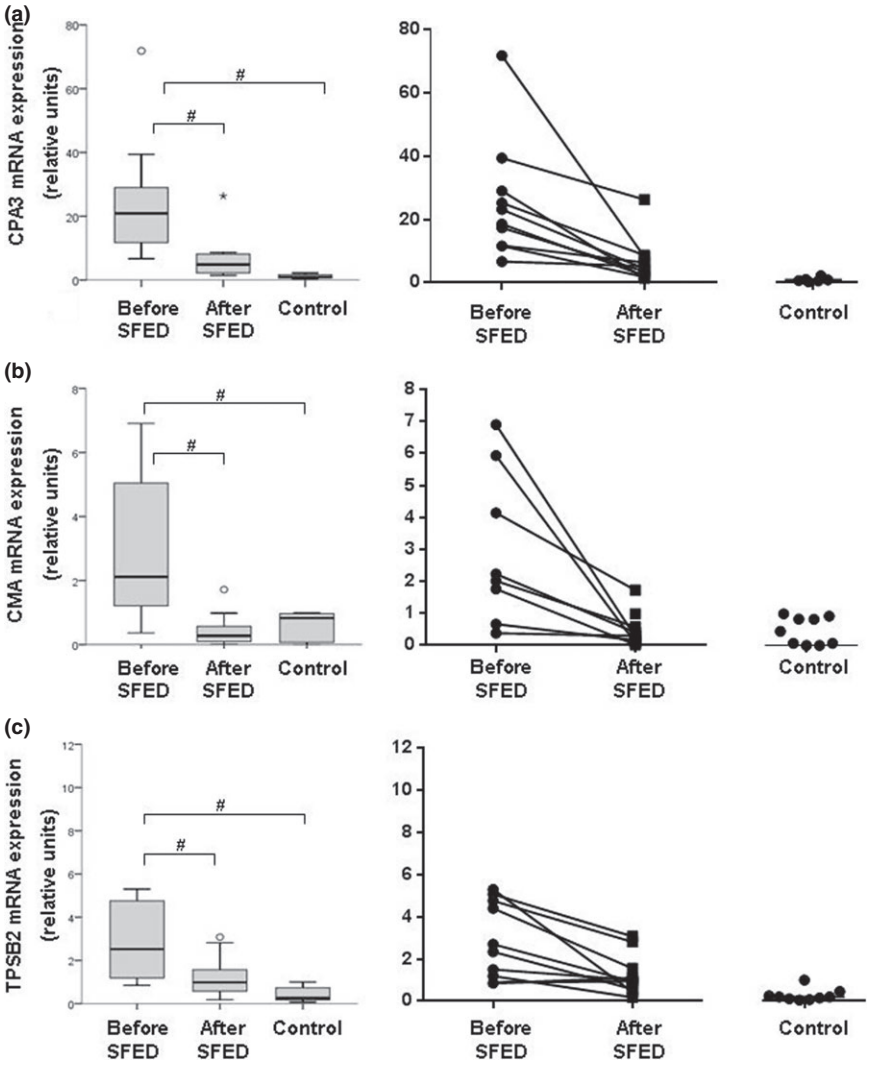


Fig. 5. Gene expression levels of the major mast cell-characteristic proteases in patients with EoE (at baseline and after six-food elimination diet [SFED]-induced remission), and in control subjects. Median and interquartile ranges are represented in the boxes, with whiskers (vertical lines) extending to a limit of ± 1.5 interquartile range. Individual changes in cytokine gene expression are provided. Horizontal bars represent means. (a) carboxypeptidase A-3 (CPA3); (b) chymase (CMA); and (c) tryptase/TPSB2). #Statistically significant differences ($P < 0.05$) before and after treatment in EoE patients compared with controls.

effect of IgE-mediated reactions [56]. Epithelial cells have been shown to have an increasing role as major effectors in initiating EoE, both through recruiting iNKT cells (a major cytokine source) towards the oesophageal epithelium, and through the release of eotaxin-3 and other chemoattractants [57, 58]. Epithelial- and mesenchymal-released TSLP is a key regulator for which a connecting role between the adaptive and innate mucosal-associated immune response has been suggested [47, 59]. In any case, the definitive exclusion of a putative role for IgE-promoting, mast cell-dependent, immediate reactions would require evidence of mast cell activation just after challenging a patient with a known food trigger for EoE, and this has yet to be demonstrated.

Our study is, to the best of our knowledge, the first to find a direct relationship between oesophageal symptoms and gene expression levels of mast cell proteases in adult EoE. Several oesophageal motor disturbances have been identified in patients with EoE by means of manometry, suggesting smooth muscle dysfunction as the origin of symptoms [60]. The ability of mast cells to induce dysmotility and visceral hyperalgesia has been repeatedly documented in several gastrointestinal inflammatory disorders [61–63], including EoE [18]. Indeed, increased mast cell counts are common in the smooth muscle of patients with EoE and have been shown to promote oesophageal smooth muscle contractility *in vitro* [18], although they decrease after topical corticosteroid therapy.

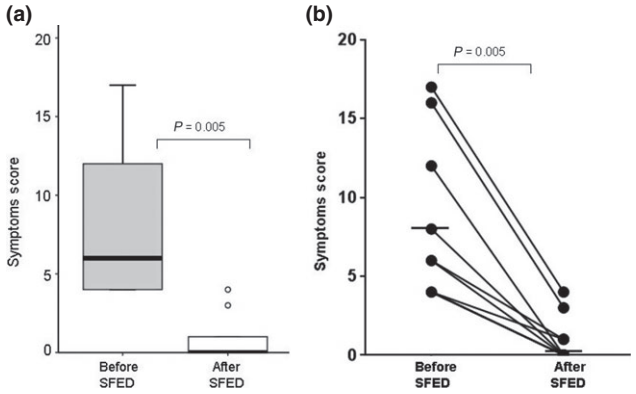


Fig. 6. Score of oesophageal symptoms in patients with EoE: patients at basal conditions and after six-food elimination diet (SFED)-induced histological remission, using the symptom score elaborated by Zaninotto et al. [40] for achalasia. (a) Medians and IQRs are represented in the boxes, with whiskers (vertical lines) extending to a limit of ±1.5 IQRs. (b) Individual changes in symptom score induced by an SFED in patients with EoE. Bars represent means.

Table 3. Relationship between mast cells, eosinophils, gene expression, and clinical symptoms score

| | Spearman's rho | *P* |
|---|---|---|
| Mast cell peak count – Carboxypeptidase 3 | 0.61 | 0.004 |
| Mast cell peak count – Chymase | 0.48 | 0.043 |
| Mast cell peak count – Tryptase | 0.47 | 0.038 |
| Mast cell peak count – Symptom Score | 0.78 | < 0.001 |
| Carboxypeptidase 3 – Symptom Score | 0.67 | 0.001 |
| Chymase – Symptom Score | 0.62 | 0.006 |
| Tryptase – Symptom Score | 0.44 | 0.049 |
| Mast cell peak count – Eosinophil peak count | 0.80 | < 0.001 |
| Eosinophil peak count – CCL26 | 0.80 | < 0.001 |
| Eosinophil peak count – Symptom Score | 0.89 | < 0.001 |
| CCL11 – Symptom Score | 0.45 | 0.045 |
| CCL26 – Symptom Score | 0.71 | 0.001 |
| SCF – Symptom Score | 0.39 | 0.085 |

The ability of dietary therapy in the form of food restriction to modify the gene expression of mast cells in the oesophageal mucosa of adult patients with EoE had previously only been assessed in a series of six adults [64]. The study found that CPA-3 expression directly correlated with that of eotaxin-3, both of which decreased after food elimination, but increased again during a food reintroduction protocol which led to disease recrudescence. Unfortunately, the researchers did not assess changes in mast cell counts. Our work thus validates previous results and provides additional evidence regarding the regulatory pathways underlying the complex relationship between eosinophils and mast cells.

One strength of our study is that it is the only one to include patients with EoE at the moment of diagnosis; thus, the subjects had no previous exposure to topical steroids or any other anti-inflammatory drugs. As such, the baseline cell densities and gene expression levels obtained can be considered a true reflection of the pathophysiological changes associated with EoE. Additionally, we have determined gene expression for mast cell-related genes by means of real-time PCR in parallel with an examination of protein expression through immunofluorescence staining, finding both to be associated with eosinophil density and symptom score.

Nevertheless, our study has several limitations. The small sample size (only 10 subjects per group) is a result of the difficulty in recruiting patients naïve to EoE therapies who also responded to an SFED. However, the strong associations between cell infiltration, gene expression levels, and oesophageal symptoms score observed in our series make us confident that the results are sufficiently strong and meet our study goals. Another limitation is that while our control group included individuals matched with patients with EoE by gender, the controls were significantly older. This is due to the fact that, according to current guidelines for managing dyspeptic symptoms, endoscopic exams can be avoided in young patients who do not present alarm symptoms. Instead, the standard strategy is to test for *Helicobacter pylori* infection through the urea breath test and then direct treatment [65]. In this sense, the difficulty in recruiting younger individuals undergoing endoscopic exams prevented us from completely matching the age of both groups. One final limitation worth mentioning is that we used a score for evaluating EoE-associated symptoms that had not actually been validated for EoE, but for achalasia. In fact, a number of scales have been used to measure oesophageal symptoms in EoE [36, 66, 67] as a validated tool for clinical assessment is still lacking [68]. In any case, our symptoms scale, which is based on the intensity and frequency of different oesophageal symptoms, has proved reliable and accurate in evaluating variations among individual patients.

In conclusion, our study characterized most oesophageal mast cells as $MC_{TC}$, which play a relevant role in the pathophysiology of EoE and its associated symptoms. It also documented the efficacy of dietary treatment in reversing the increased density and activity of these cells. Future studies should define the exact mechanisms of mast cell activation and their complex interactions with other inflammatory cells in the pathophysiology of EoE.

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## Conflicts of interest

The authors declare no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Clinical characteristics of control subjects included in the study.

**Table S2.** Genes included in the study.