Changes in IgG4 and IL-10 expression in adults with eosinophilic esophagitis on a two-food elimination diet

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Abstract

Background: Eosinophilic esophagitis (EoE) is increasingly diagnosed in patients with dysphagia and upper gastroenteric symptoms. Elimination diets and/or pharmacologic agents may accomplish temporary remission, but long-term control is challenging. Type-2 immunity to ingested antigens can induce EoE histopathology via non-IgE-dependent mechanisms, possibly involving IgG4 and IL-10 production. To elucidate the contribution of IgG4- and IL-10-producing cells to EoE pathogenesis, we examined their frequencies and association with clinical and histologic endpoints in adult EoE patients given a two-food elimination diet (TFED).

Methods: Sixteen patients with EoE were prescribed a TFED. Biopsies collected at baseline and follow-up were used for immunofluorescent detection of IgG4- and IL-10-expressing cells and serum food-specific IgG4 were measured. All variables were correlated with established histologic measures of disease activity.

Results: Patients exhibited significant clinical improvement and significant reduction in esophageal eosinophilia and overall histology. A significant decrease in the frequencies of IL-10-expressing cells was also observed, which correlated with histologic changes. In contrast, a concomitant decline in serum and esophageal IgG4, while substantial, did not correlate with IL-10 \( ^{+} \)-cell frequencies or any histologic parameter of EoE activity.

Conclusions: The close association of esophageal IL-10 expression with histologic features and their changes after a TFED suggests a critical role of this cytokine in EoE pathogenesis. Conversely, IgG4 serum and mucosal expression, while reflecting the level of exposure to relevant food antigens, is not obviously related to EoE histopathology or IL-10 expression. Studies are needed to characterize IL-10 cellular sources and their functions in EoE progression and treatment response.

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Running Title: Diet-induced IgG4 and IL-10 changes in eosinophilic esophagitis

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CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
All authors participated in the study. D.G., V.C., and P.I. designed the study. R.A., A.C., L.R., M.G., A.D., R.D., and Va.C. developed the methodology. D.G., F.D., A.S., B.S, M.G., and P.I. characterized and recruited the study subjects. R.A., A.C., F.D., L.R., B.S., L.C., and Va.C. collected the data. R.A., D.G., A.C., L.R., V.C., and P.I. analyzed the data. V.C. and P.I. prepared the manuscript. R.A., D.G., A.C., Va.C., V.C., and P.I. reviewed the manuscript. All approved the final version for submission.

ABBREVIATIONS
BMI, body mass index; BZH, basal zone hyperplasia; DAPI, 4’,6-diamidino-2-phenylindole; EGD, esophagogastroduodenoscopy; EoE, eosinophilic esophagitis; EI, eosinophilic inflammation; EoEHSS, EoE histologic scoring system; GC, glucocorticoid; HPF, high-power field; IQR, interquartile range; PBS, phosphate-buffered saline; PEC, peak eosinophil count; PPI, proton pump inhibitor; SEA, surface epithelial alteration; SFED, six-food elimination diet; sIgG4, specific IgG4; Sρ, Spearman’s rank correlation coefficient; TFED, two-food elimination diet; Th, T follicular helper; WSR, Wilcoxon signed-rank

KEYWORDS
diet, eosinophils, esophagus, IgG4, IL-10

ABSTRACT
Background: Eosinophilic esophagitis (EoE) is increasingly diagnosed in patients with dysphagia and upper gastrointestinal symptoms. Elimination diets and/or pharmacologic agents may accomplish temporary remission, but long-term control is challenging. Type-2 immunity to ingested antigens can induce EoE histopathology via non-IgE-dependent mechanisms, possibly involving IgG4 and IL-10 production. To elucidate the contribution of IgG4- and IL-10-producing cells to EoE pathogenesis, we examined their frequencies and association with clinical and histologic endpoints in adult EoE patients given a two-food elimination diet (TFED).
Methods: Sixteen patients with EoE were prescribed a TFED. Biopsies collected at baseline and follow-up were used for immunofluorescent detection of IgG4- and IL-10-expressing cells and serum food-specific IgG4 were measured. All variables were correlated with established histologic measures of disease activity.

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INTRODUCTION

Eosinophilic esophagitis (EoE), whose prevalence has risen in recent years, is today commonly diagnosed in children experiencing feeding problems, abdominal pain and vomiting or adults with dysphagia and impaction.1,2 Symptoms may mimic reflux disease and esophagogastroduodenoscopy (EGD) is required for patients’ assessment.3 EGD can visualize inflammatory changes, e.g. exudates and edema, and, especially in scarcely controlled adults, remodeling features, e.g. rings, fragility, and narrowing.4 Sampling of the mucosa allows the definition of eosinophil-rich infiltrates and possible signs of remodeling, e.g. basal zone hyperplasia (BZH) and dilated intercellular spaces.5 Disease activity is defined by the detection, within the epithelium, of [?]?15 eosinophils/high-power field (HPF), excluding other causes of esophageal eosinophilia.6

EoE pathogenesis results from the interaction of genetic and extrinsic factors, including food antigen load.7 The main treatment options are dietary restrictions, proton pump inhibitors (PPI), swallowed glucocorticoids (GC), and endoscopic dilation.11 While avoidance of certain foods may relieve the clinical and histologic picture in most patients, flares may reoccur upon reintroduction.12-16 Variable combinations of dietary and pharmacologic treatments do not accomplish long-term disease control, and no consistent predictors of response have been described.17,18 Emerging type-2 immunity-targeted therapies, which have proven effective in IgE-mediated diseases, represent a promising option.11,19 Type-2 immunity to food and inhaled allergens, via the release of IL-4, IL-5, and IL-13, may lead to barrier dysfunction, inflammatory cell recruitment, and remodeling.10 Frequently comorbid atopy, e.g. allergic rhinitis, chronic rhinosinusitis, asthma, and food allergy,8,9 along with raised levels of Th2 cytokines and the abundance of eosinophils and mast cells in the esophageal mucosa, led to speculation that EoE could be a bona fide allergic condition.20,21 However, allergy diagnostics does not necessarily predict trigger foods in adults with EoE, and targeting IgE-dependent responses with mast cell stabilizers or FcεRI antagonists is scarcely effective.22-24

IgG4, an Ig class associated with type-2 responses,24,25 has been related to disease activity and assigned a diagnostic value in different conditions.26-28 IgG4 deposits and IgG4-expressing cells in the esophageal mucosa, along with elevated titers of specific IgG4 (sIgG4) in sera and esophageal secretions, have been consistently documented in EoE.29-32 However, the regulation of IgG4 expression and, more cogently, their contribution to pathogenesis remain open questions. Cells expressing IL-10, a cytokine directing IgG4 switching in activated B cells,33 have been detected at higher frequencies in the EoE mucosa.20,34 Intriguingly, though, expansion of IL-10-producing T and B cells and increased levels of sIgG4 for relevant allergens are established markers of immune tolerance.35 To gain further knowledge of the possible contribution of IgG4- and IL-10-producing cells to EoE pathogenesis, we have conducted a study exploring their association with clinical and histologic endpoints in adult Southern Italian EoE patients given a two-food elimination diet (TFED). We hypothesized that patients experiencing remission of the clinical and histologic picture would exhibit a consistent decline in the expression of these putative markers of disease activity.

METHODS
2.1 | Patients

Adults with symptoms of esophageal dysfunction were observed in 2019-21 at the Gastroenterology Unit of San Giovanni di Dio e Ruggi d’Aragona University Hospital in Salerno, Italy. EoE was diagnosed by detecting 15/HPF (400x magnification) peak eosinophil counts (PEC) in esophageal biopsies collected by EGD excluding other causes of esophageal eosinophilia.\(^6\) Sixteen consecutive patients were prescribed a TFED (excluding wheat and milk) by a specialized dietician, reassessed, and subjected to EGD with biopsies 90 d thereafter. Before and after treatment patients completed a questionnaire scoring the intensity and frequency of upper gastrointestinal symptoms, yielding a compound score of 0-30.\(^36\) Patients consented to use biopsy and blood specimens for research as approved by the Ethical Committee of Azienda Sanitaria Locale Napoli 3 Sud (determination #35, 03/18/2020).

2.2 | Esophageal biopsies and immunohistology

Biopsies were fixed in 10% neutral-buffered formalin and embedded in paraffin, then cut into 3.5-μm-thick sections, stained with hematoxylin-eosin, and analyzed by two blinded pathologists by light microscopy (Olympus BX43). PEC were determined within the HPF with the highest eosinophil density, one HPF being a 0.237-mm\(^2\) circular field with a 40x objective and 10x oculars. Additional features were scored for grade (severity) and stage (extent) using a validated EoE histologic scoring system (EoEHSS).\(^5,37\) Five-μm sections from distal biopsies were deparaffinized, antigen-retrieved, washed, and blocked with 1% bovine albumin for 2 h at RT. Fixed sections were incubated at RT with rabbit anti-human IgG4 (Abcam), rat anti-human IL-10 (Invitrogen), or isotype controls, for 1 h, then Alexa 647-F(ab')\(_2\) donkey anti-rabbit IgG (H+L) and Alexa 488-F(ab')\(_2\) goat anti-rat IgG (H+L) (Jackson) for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:10,000; Invitrogen) for 5 min. Fluorescent cells were enumerated by two blinded investigators by confocal microscopy using a 40x objective (TCS SP5 II; Leica), and frequencies averaged from five non-overlapping HPF.

2.3 | Measurements of serum sIgG4

Blood samples from patients at baseline and follow-up were separated (500g, 10 min) and stored at -80°C. Sera were assayed for sIgG4 for cow’s milk and wheat components or extracts using the ImmunoCAP protocol (Thermo Fisher).

2.4 | Statistical analysis

Data were analyzed and graphed using Prism 7.0 (GraphPad). Most variables resulted arranged in a non-normal, positively skewed distribution. Therefore, medians and interquartile ranges (IQR) were calculated, and a two-tailed Wilcoxon signed-rank (WSR) test was used for pair-wise comparisons. Associations between variables were analyzed by the Spearman’s rank \(\rho\) correlation coefficient (S\(\rho\)). The level of significance was set at \(P < 0.05\).

3 | RESULTS

3.1 | Characterization of the study patients

Table 1 summarizes the demographic and clinical characteristics of the patients enrolled. Patients were aged 18-66 y (36.50, 23.50-53.00), were predominantly (75%) male, and ~70% had a history of atopic illness, mostly asthma and/or rhinitis. Dysphagia was reported by >80% of patients, other symptoms being reported less frequently, and 2/16 reported no symptoms, presumably due to coping strategies to facilitate food ingestion. Intensity-frequency scores for all symptoms ranged 0-17 (7.00, 4.00-10.75).

Following the initial evaluation, patients were subjected to EGD with biopsies, and blood collected, for baseline determinations of histologic and serologic endpoints. Patients were then given a TFED and reassessed after 90 d (127.00 d, 97.50-139.30). Collectively, patients reported a significant clinical improvement at follow-up, with a symptom score of 0.00 (0.00-5.75; \(P =0.0006\)). Specifically, significant declines were seen in the intensity (\(P =0.0054\)) and frequency (\(P =0.0032\)) of dysphagia for solids, but not in less common symptoms. However, 4/16 patients experienced partial (<50.00%) or no clinical response. To investigate the
pathophysiologic basis of this heterogeneity and objective markers of response and adherence to treatment, samples collected before and after the diet, de-identified and assigned random codes, were blindly analyzed at completion of the trial.

3.2 | Histologic response to the diet

Before initiating the diet, all patients had >15/HPF PEC in esophageal biopsies, ranging 22-317/HPF (33.00, 45.00-109.50). Listed in Table 2 are histologic parameters of disease activity, scored by grade and stage, and their cumulative score, referred to as EoEHSS, which ranged 12-39 at baseline (24.00, 16.25-28.00). At follow-up PEC ranged 0-98/HPF (33.00, 0.00-44.25; \( P = 0.0074 \)), while the EoEHSS ranged 0-39 (15.50, 1.75-20.50; \( P = 0.0301 \)) (Figs. 1A-B). A significant decline was appreciated in eosinophil inflammation (EI)-stage, BZH, eosinophil surface layering, and surface epithelial alteration (SEA), but not in other EoEHSS components (Table 2). Thus, we observed significant reductions of validated histologic parameters of disease activity, consistent with the overall clinical improvement. However, the degree of improvement varied considerably across patients (Fig. 1C), whereby 6/16 (37.50%) exhibited a complete histologic response, or <15/HPF PEC, and another five (31.25%) had a partial response, or >15/HPF PEC but \( \leq 50\% \) reduced from baseline.14

Conversely, the EoEHSS decreased \( \leq 50\% \) in 7/16 patients (43.75%), with only four (25.00%) showing a complete response, possibly reflecting more stringent disease assessment criteria using this scoring system.5,37 Moreover, while there was a good concordance between baseline PEC and EoEHSS (Fig. 1D) and their response to treatment (Fig. 1E), in few, yet remarkable cases (color coded in Figs. 1C-E), diverging responses were appreciated in some EoEHSS components, mostly related to epithelial dysfunction or remodeling, e.g. lamina propria fibrosis, rather than EI (not shown). Regardless, none of these parameters correlated with the clinical intensity-frequency score before or after treatment or its percent variation (not shown).

3.3 | Changes in IgG4 and IL-10 expression in the esophageal mucosa

We next assessed whether the histologic response in EoE patients following a TFED was paralleled by changes in the frequencies of cells expressing IgG4 and IL-10. IgG4 and IL-10 were mostly associated with distinct cells (Fig. 2A), with only a small proportion expressing both markers in a minority of patients (3/16). Regardless, \( \geq 0.1\% \) IgG4+ cells were seen in baseline samples from 8/16 patients, their median frequency being 1.15% (0.00-4.34%), which decreased to 0.00% (0.00-1.22%) at follow-up (Fig. 2B), a change that, while substantial, only approximated significance (\( P = 0.0547 \)). IL-10 was found on higher proportions of cells from most patients at baseline (13/16), with median frequencies of 5.52% (2.59-8.41%), and these were significantly reduced after treatment, down to 2.71% (0.78-4.05%; \( P = 0.0250 \)) (Fig. 2C).

To understand the pathophysiologic significance of these findings, a possible association with established histologic features was sought by statistical analysis. Baseline PEC showed a significant, direct correlation with the frequencies of IL-10+ (\( S_\varphi = 0.57; P = 0.0243 \)), but not IgG4+ cells (\( S_\varphi = 0.02; P = 0.9420 \)) (Figs. 2D-E). A similar trend was seen with baseline EoEHSS, as a positive correlation, yet not reaching significance (\( S_\varphi = 0.38; P = 0.1499 \)), was only apparent with IL-10+ -cell frequencies, resulting from a significant association with EI-grade (\( S_\varphi = 0.57; P = 0.0275 \)). No correlation was found between IgG4+ -cell frequencies and the EoEHSS (\( S_\varphi = 0.19; P = 0.4776 \)) or any of its components. Moreover, we found no significant correlations between IgG4+ - vs. IL-10+ -cell frequencies before (\( S_\varphi = 0.24; P = 0.3589 \)) or after treatment (\( S_\varphi = 0.14; P = 0.5952 \)). Finally, no significant correlations were seen between these immune variables and histologic parameters at follow-up (not shown). However, diet-associated changes (expressed as percent from baseline) of IL-10+ -cell frequencies showed a significant direct correlation with concomitant changes of PEC (\( S_\varphi = 0.72; P = 0.0007 \)) or EoEHSS (\( S_\varphi = 0.63; P = 0.0235 \)) (Figs. 3A-B). A significant inverse correlation was also seen between baseline frequencies of IL-10+ cells with the percent changes of the EoEHSS (\( S_\varphi = -0.54; P = 0.0316 \)), but not with the corresponding PEC variations (\( S_\varphi = -0.20; P = 0.4625 \)) (Figs. 3C-D).

No such connections were apparent between IgG4+ -cell frequencies and the histologic response or between any of these variables, the clinical response, and the clinical and demographic features (not shown). One notable exception were the body mass indexes (BMI) (Figs. 3E-F), which, at baseline, showed a strong, significant
inversive correlation with PEC ($r_p=-0.72; P =0.0023$) and IL-10+ cell frequencies ($r_p=-0.69; P =0.0037$), but not with the EoEHSS ($r_p=-0.10; P =0.7237$) or IgG4+ cell frequencies ($r_p=0.33; P =0.2167$). Such associations were not observed at follow-up (not shown).

3.4 | Changes in serum sIgG4 for food antigens

To further elucidate the contribution of IgG4 to disease activity, we measured levels of sIgG4 for excluded milk and wheat antigens in sera collected before and after a TFED. At baseline, 10/16 patients (62.50%) had $[?5\mu g/mL sIgG4 for milk components Bos d 4, 5, and/or 8, and the same number, but not necessarily the same patients, had similar levels of sIgG4 for wheat antigen and/or the Tri a 14/19 components. Levels of all these antibodies, except those against Tri a 19, significantly declined after treatment (Table 3). Levels of milk sIgG4 correlated significantly with each other or with the frequencies of esophageal IgG4+ cells before or after treatment. Shown in Fig. 4A is the significant correlation ($r_p=0.51; P =0.0444$) between Bos d 4 sIgG4 and IgG4+ cell frequencies at baseline. In contrast, no consistent associations were found among wheat sIgG4 (not shown) or between these antibodies and milk sIgG4 or esophageal IgG4+ cells (Fig. 4B).

Finally, serum levels of milk or wheat sIgG4, at baseline or follow-up, or their changes across the treatment, while showing a similar trend, did not correlate with esophageal IL-10+ cell frequencies (Figs. 4C-D), PEC, or EoEHSS (not shown).

4 | DISCUSSION

Empiric elimination of foods most commonly associated with EoE manifestations is today widely adopted in children and adults. Current strategies include six-food elimination diets (SFED) followed by stepwise reintroduction to identify specific triggers, or scaling up a TFED depending on the initial response. As these protocols are time- and resource-consuming and rely on patients’ adherence, identifying objective measures of response would be desirable. This study, the first conducted in Southern Italy, has investigated clinical, histopathologic, and immune endpoints in adults with EoE prescribed a TFED excluding dairy and gluten-containing foods, which are most associated with recurrence upon reintroduction. We have observed a significant reduction of established histologic parameters of EoE activity, and ~40% patients exhibited complete remission, in line with studies of patients on a TFED without concomitant pharmacologic therapy. There was a good concordance between PEC and EoEHSS values at baseline and follow-up, and their response to treatment, confirming recent findings in patients treated with a SFED or swallowed GC. Few notable exceptions possibly reflect the more complex nature of the EoEHSS, implying that distinct mechanisms may be involved in EI and concurring changes in the mucosal architecture. Moreover, adequate sampling of deeper mucosal layers is required to assess such EoEHSS parameters as BZH or fibrosis. Regardless, the lack of concordance of histologic and clinical parameters of response is in agreement with studies scoring self-reported symptoms.

These limitations stress the need for independent measures of disease activity, response, and adherence to treatment. An expanding literature supports the possible role of IgG4, IL-10 and their cellular sources in EoE pathogenesis. However, limited information is available on their relationship with more established parameters of disease activity and remission. Increased total and sIgG4 in sera and esophageal secretions, esophageal IgG4 and IgG4-expressing cells have been consistently reported in adult and pediatric EoE. We detected esophageal IgG4, mostly in association with cellular elements, in 50% of baseline samples, in line with studies documenting higher frequencies of IgG4+ cells in the EoE mucosa. We observed an almost significant decrease in their frequencies at follow-up (Fig. 2B), which was paralleled by a significant decrease of serum sIgG4 for excluded food antigens (Table 3). To our knowledge, this is the first documentation of the response of these combined endpoints to an exclusive dietary regimen in adults with EoE. While Weidlich et al. documented a significant reduction in esophageal IgG4+ plasma cells following treatment with topical GC, no such differences were seen in trials variably combining dietary and pharmacologic treatments. Regardless, while we found a significant correlation between serum milk sIgG4 titers and mucosal IgG4+ cell frequencies (Fig. 4A), neither variable correlated with the corresponding PEC or EoEHSS (Fig. 2D), confirming earlier studies seeking an association between these variables and their responses to treatment. We also found at follow-up significantly lower serum sIgG4 for wheat
or Tri a 14, a common food allergen in Southern Italy.\textsuperscript{46} but not for Tri a 19 (ω-5 gliadin). Differently from milk sIgG4, these did not correlate with esophageal IgG4\textsuperscript{+}-cell frequencies or other parameters of disease activity (Fig. 4). Increased levels of milk and wheat sIgG4 have been found in the esophageal secretions from active EoE.\textsuperscript{31,47} Preferential uptake of milk antigens was seen in the adult EoE mucosa, whereas gliadin penetration was more critically dependent on barrier perturbation and disease activity.\textsuperscript{48} Thus, IgG4 production in response to distinct milk or wheat components may follow diverging trajectories and associate with distinct clinical phenotypes, as shown in IgE-dependent conditions.\textsuperscript{39,50} The dynamics of IgG4 generation and effector function is still largely unknown and demands more work in suitable models.\textsuperscript{28,51}

Regardless, IgG4 production in EoE, while related to food antigen intake, could be a side occurrence rather than an active player of the ensuing histopathology in at least some cases.\textsuperscript{43}

The reduced frequencies of esophageal IL-10-expressing cells after a TFED (Fig. 2C) is in line with a study measuring IL10 transcripts in EoE biopsies after a four-food elimination diet.\textsuperscript{52} Earlier work showed that IL10 and IGHG4 expression correlated in pediatric EoE and were in turn related to histologic variables.\textsuperscript{26} In contrast, we did not observe a relationship between the frequencies of IL-10\textsuperscript{+} and IgG4\textsuperscript{+} cells in baseline or follow-up biopsies, and only IL-10\textsuperscript{+} cells correlated with PEC or EoEHSS determinations (Fig. 2E). Further, we observed a direct correlation between diet-associated changes of PEC or EoEHSS and IL-10\textsuperscript{+}, but not IgG4\textsuperscript{+} cell frequencies (Figs. 3A-B). Intriguingly, variations of EoEHSS, but not PEC, were inversely related to the baseline frequencies of IL-10\textsuperscript{+} cells (Figs. 3C-D), implying that a higher esophageal expression of IL-10 would predict a more profound tissue response. Despite a direct relationship of IL-10\textsuperscript{+}-cell frequencies with EI measures, this inverse association was apparently driven by independent parameters, e.g. SEA-grade, which underwent the most significant decline among EoEHSS components (Table 2). SEA was identified as a distinctive alteration in an EoE endotype characterized by more severe fibrostenosis in non-atopic adults and low expression of genes involved in epithelial cell differentiation and barrier function.\textsuperscript{53} Thus, a higher prevalence of IL-10-expressing cells could be associated with an EoE phenotype that responds more promptly to elimination diets, as hinted at in a study showing that esophageal IL10 expression associated with a “food impaction” phenotype, whereas genes responsible for barrier integrity were dysregulated in a fibrotic, more refractory phenotype.\textsuperscript{52}

The contribution of IL-10 to disease activity is suggested in studies linking EoE histopathology to the accumulation of IgG4, an antibody class whose switch is promoted by this cytokine.\textsuperscript{33} IGHE and IGHG4 germline transcripts, indicia of IgE and IgG4 switching, have been documented in the esophageal mucosa of EoE patients.\textsuperscript{54} Thus, the EoE mucosa may act as an inductive site where T follicular helper (Tfh) cells promote B-cell clonal responses to food antigens.\textsuperscript{48} This theory needs validation in studies showing IL-10-expressing Tfh cells in ectopic germinal centers within the EoE mucosa, as documented in IgG4-related diseases.\textsuperscript{53} Regardless, our findings speak against the idea that IgG4 induction is a univocal consequence of IL-10 expression in the EoE mucosa. No statistical association was in fact apparent between the two variables, and negligible frequencies of IgG4\textsuperscript{+} cells, with correspondingly low serum sIgG4 levels, were found in ~50% of specimens harboring substantial numbers of IL-10\textsuperscript{+} cells. Moreover, the exclusive association with the histologic picture and its response to diet suggests that IL-10 role in disease activity may transcend IgG4 induction and depends on some other effector functions.

The close concordance of IL-10 expression with measures of eosinophilia has no precedents in human or animal studies.\textsuperscript{56,57} In fact, agents that promote IL-10 production, e.g. butyrate, can downregulate eosinophile-specific chemokines and eosinophil trafficking and restore IL-13-disrupted barrier function in the esophageal mucosa.\textsuperscript{58} A tight link between IL-10 expression and eosinophilia is further supported by their independent, reverse association with the patients’ BMI (Figs. 3E-F). Obesity has been identified as a risk factor for EoE in population studies and animal models,\textsuperscript{59,60} and obese EoE patients may be less responsive to topical GC.\textsuperscript{61} However, studies in established EoE cases indicate that a lower BMI is associated with narrowing, a reduced response to PPI and an increased need for repeat dilation.\textsuperscript{62-64} The reasons for this apparent discrepancy are unclear and may reflect the relative prevalence of distinct EoE phenotypes.\textsuperscript{52,53} Regardless, in no study to date has the effect of elimination diets been stratified by BMI or related variables. Based on our findings, we would envision that a lower BMI, being associated with higher numbers of IL-10\textsuperscript{+} cells in
the esophageal mucosa, would in turn be predictive of a more substantial histologic response to the diet.

IL-10, a signature Treg cytokine, is a regulatory factor downplaying innate and adaptive inflammatory responses. Increased FoxP3+ Treg-cell numbers were documented in the esophagus and blood of children with EoE, but opposite findings were reported in EoE adults. It is possible that in at least some patients esophageal IL-10 is associated with other cell sources, including B regulatory cells and myeloid cells. Higher frequencies of FoxP3+ eosinophils have also been demonstrated in adults with EoE, but the immunoregulatory functions of these cells have yet to be elucidated. Animal studies of food allergy and single-cell profiling of the local T-cell landscape in allergic disease converge on the idea that IL-10 expression in the EoE mucosa may trace to Treg cells recruited along with Th2 cells by epithelial alarmins, but eventually transitioning to a Th2-like phenotype in a Th2-biased milieu. IL-10 production and eosinophil trafficking might then represent two faces of the same coin, whereby an initial homeostatic response to as yet unidentified insults to the epithelium would progress into remodeling and fibrosis in the context of food antigen-driven, sustained Th2 responses. The dominant role of Th2-type cytokines is supported in clinical trials of dupilumab, an IL-4/IL-13 antagonist approved for EoE treatment in the US. Conversely, agents targeting the IL-5/eosinophil axis, while approved for certain allergic conditions, have shown limited effectiveness in EoE. Therefore, eosinophils, while proxies of Th2-driven immunity, may not be directly responsible for the associated histopathology.

This study, while limited in scope and insufficiently powered for group-wise analyses of differing clinical and histologic responses, provides some clues about the possible roles of IgG4 and IL-10 in EoE. Our findings, while dismissive of a pathogenic function of IgG4-producing cells, are consistent with the idea that IL-10 is expression of a protective, if transient, response to epithelial damage, aimed at preventing progression in the context of a robust Th2 response. Controlled trials of adequately powered cohorts of well-characterized EoE phenotypes are awaited to validate these theories and help resolve the controversial aspects of this condition’s pathophysiology.

Word count: 3540

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**Table 1.** Demographic and clinical features of the 16 patients enrolled in this study. Values shown are medians (IQR) unless otherwise indicated.

<table>
<thead>
<tr>
<th>Male gender (%)</th>
<th>12 (75.00)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>36.50 (22.50-53.00)</td>
</tr>
<tr>
<td>Body mass index (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>24.50 (22.25-25.88)</td>
</tr>
<tr>
<td>Comorbid atopy (%)</td>
<td>9 (56.25)</td>
</tr>
<tr>
<td>Presenting symptoms (%)</td>
<td>13 (81.25)</td>
</tr>
</tbody>
</table>

**Table 2.** Medians (IQR) of the indicated histologic parameters, assessed by grade (G) or stage (S), in the esophageal biopsies collected from 16 EoE patients before (baseline) and after a TFED (follow-up). Differences were assessed for significance (*P*) using the WSR test.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Follow-up</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophilic inflammation (EI)</td>
<td>G 2.00 (2.00-3.00)</td>
<td>2.00 (0.00-2.75)</td>
<td>0.0735</td>
</tr>
<tr>
<td></td>
<td>S 3.00 (3.00-3.00)</td>
<td>2.00 (0.00-3.00)</td>
<td>0.0078</td>
</tr>
<tr>
<td>Basal zone hyperplasia (BZH)</td>
<td>G 2.00 (2.00-2.75)</td>
<td>1.00 (0.00-2.00)</td>
<td>0.0166</td>
</tr>
<tr>
<td></td>
<td>S 2.00 (2.00-3.00)</td>
<td>1.00 (0.00-2.00)</td>
<td>0.0210</td>
</tr>
<tr>
<td>Eosinophil abscess (EA)</td>
<td>G 1.00 (0.00-1.75)</td>
<td>1.00 (0.00-1.75)</td>
<td>0.7456</td>
</tr>
<tr>
<td></td>
<td>S 1.00 (0.00-2.00)</td>
<td>1.00 (0.00-2.00)</td>
<td>0.5645</td>
</tr>
<tr>
<td>Eosinophil surface layering (ESL)</td>
<td>G 1.00 (0.25-1.75)</td>
<td>0.00 (0.00-0.00)</td>
<td>0.0088</td>
</tr>
</tbody>
</table>
Table 3. Median (IQR) serum levels (μg/mL) of IgG4 specific for the indicated milk or wheat antigens in 16 patients before (baseline) and after a two-food elimination diet (follow-up). Differences were tested for significance ($P$) by pair-wise comparison using the WSR test.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Baseline</th>
<th>Follow-up</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S 1.50 (0.25-2.00)</td>
<td>0.00 (0.00-0.00)</td>
<td>0.0166</td>
</tr>
<tr>
<td>Dilated intercellular spaces (DIS)</td>
<td>G 3.00 (2.00-3.00)</td>
<td>2.50 (0.00-3.00)</td>
<td>0.4468</td>
</tr>
<tr>
<td></td>
<td>S 2.00 (2.00-3.00)</td>
<td>1.50 (0.00-3.00)</td>
<td>0.1152</td>
</tr>
<tr>
<td>Surface epithelial alteration (SEA)</td>
<td>G 1.00 (1.00-2.00)</td>
<td>0.00 (0.00-1.00)</td>
<td>0.0049</td>
</tr>
<tr>
<td></td>
<td>S 2.00 (1.00-2.00)</td>
<td>0.00 (0.00-1.00)</td>
<td>0.6250</td>
</tr>
<tr>
<td>Dyskeratotic epithelial cell (DEC)</td>
<td>G 0.00 (0.00-0.75)</td>
<td>0.00 (0.00-0.00)</td>
<td>0.6250</td>
</tr>
<tr>
<td></td>
<td>S 0.00 (0.00-0.75)</td>
<td>0.00 (0.00-0.00)</td>
<td>0.6250</td>
</tr>
<tr>
<td>Lamina propria fibrosis (LPF)</td>
<td>G 0.00 (0.00-1.50)</td>
<td>0.00 (0.00-1.75)</td>
<td>0.6563</td>
</tr>
<tr>
<td></td>
<td>S 0.00 (0.00-1.50)</td>
<td>0.00 (0.00-2.75)</td>
<td>0.7656</td>
</tr>
<tr>
<td>EoEHSS</td>
<td>G 11.00 (8.00-12.75)</td>
<td>7.50 (1.00-9.75)</td>
<td>0.0588</td>
</tr>
<tr>
<td></td>
<td>S 12.00 (9.25-15.50)</td>
<td>7.50 (0.75-10.75)</td>
<td>0.0257</td>
</tr>
</tbody>
</table>

Figure Legends

Figure 1. Histologic changes in the esophageal mucosa from EoE patients on a TFED. (A) PEC and (B) EoEHSS before and after treatment. Boxes span the median and IQR (25-75%), and whiskers the min-max range, of the indicated values in 16 patients subjected to EGD with biopsies at baseline (BL) and follow-up (FU). $P$ as indicated relative to baseline (WSR test). (C) PEC and EoEHSS values at follow-up are represented as percent of change from baseline. Each dot represents an individual patient, with medians shown as horizontal lines. Represented with purple dots are fully responsive patients, defined by PEC < 15/HPF after treatment; light blue dots are partially responsive patients, where PEC is > 15/HPF but > 50% lower than baseline; and orange dots are non-responsive patients. (D) Correlation of PEC and EoEHSS values at baseline and (E) as a percent change from baseline after the diet. Shown are the Spearman’s $\rho$ coefficients ($S_{\rho}$) and the levels of significance ($P$). Dots in (E) and in the following figures are color-coded per PEC changes shown in (C). Please note that the dot at the origin of the axes is representative of four overlapping values.

Figure 2. Immunofluorescence of cell-associated IL-10 and IgG4 in esophageal biopsies from adult EoE patients. (A) Representative fields (out of five) captured by confocal microscopy of slides from baseline biopsies of an individual patient. IL-10 and IgG4 were labeled as indicated and nuclei visualized for total cell enumeration using the DNA stain, DAPI. (B) Frequencies of IgG4$^+$ and (C) IL-10$^+$ cells before and after a TFED. Boxes span the median and IQR (25-75%), and whiskers the min-max range, of the indicated values in 16 patients at baseline (BL) and follow-up (FU). The indicated $P$ are relative to baseline (WSR test). Correlations of PEC with IgG4$^+$ (D) or IL-10$^+$ (E) cell frequencies (% of DAPI$^+$) at baseline. Dots are color-coded per PEC changes shown in Fig. 1C. Please note that a dot at (45, 0) in (D) results from two overlapping values. Shown are the Spearman’s $\rho$ coefficients ($S_{\rho}$) and the relative levels of significance ($P$).
Figure 3. The frequencies of IL-10+ cells in the EoE mucosa correlate with the histologic response to a TFED and with the BMI. Correlation of diet-associated changes of IL-10+ cell frequencies (% of DAPI+) with concomitant changes of PEC (A) or EoEHSS (B), expressed as percent changes from baseline values in n = 13 patients with baseline >1.00% IL-10+ cells. Correlation of baseline IL-10+ cell frequencies with PEC (C) or EoEHSS changes (D). Correlations of the BMI with the corresponding PEC (E) or the frequencies of IL-10+ cells (F) in EoE patients at baseline. Dots are color-coded as in Fig. 1C. Shown are the Spearman’s ρ coefficients (Sρ) and the associated levels of significance (P).

Figure 4. Serum levels of milk, but not wheat-specific IgG4 correlate with the frequencies of cells expressing IgG4 in the esophageal mucosa. Correlation of serum IgG4 specific for milk allergen component Bos d 4 (μg/mL) with the frequencies of esophageal IgG4+ (A) or IL-10+ cells (B), expressed as % of DAPI+, in EoE patients prior to a TFED. Baseline levels of milk- (C) or wheat-specific IgG4 (D) were also tested for correlation with the corresponding IL-10+ cell frequencies. Dots are color-coded as in Fig. 1C. Shown are the Spearman’s ρ coefficients (Sρ) and the P levels of significance.
Figure 1_Appanna et al.
Figure 2_Appanna et al.
Figure 3_Appanna et al.
Figure 4_Appanna et al.