

ORIGINAL ARTICLE

Gastrointestinal Diseases

Esophageal IgG4 levels correlate with histopathologic and transcriptomic features in eosinophilic esophagitis

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Abstract

Background: Recent data associate eosinophilic esophagitis (EoE) with IgG4 rather than IgE, but its significance and function have not been determined. Our aims were to measure esophageal IgG4 levels and to determine functional correlations as assessed by histologic and transcriptome analyses.

Methods: This case-control study included pediatric subjects with EoE (≥ 15 eosinophils/HPF) and non-EoE controls. Protein lysates were analyzed for IgA, IgM, and IgG1-IgG4 using the Luminex 100 system; IgE was quantified by ELISA. Esophageal biopsies were scored using the EoE histology scoring system. Transcripts were probed by the EoE diagnostic panel, designed to examine the expression of 96 esophageal transcripts.

Results: Esophageal IgG subclasses, IgA, and IgM, but not IgE, were increased in subjects with EoE relative to controls. The greatest change between groups was seen in IgG4 (4.2 mg/g protein [interquartile range: 1.0-13.1 mg/g protein] vs 0.2 mg/g protein [0.1-0.9]; $P < .0001$). Tissue IgG4 levels correlated with esophageal eosinophil counts ($P = .0006$); histologic grade ($P = .0011$) and stage ($P = .0112$) scores; and *IL4*, *IL10*, *IL13*, but not *TGFB1*, expression and had strong associations with a subset of the EoE transcriptome. Esophageal IgG4 transcript expression was increased and correlated with IgG4 protein levels and *IL10* expression.

Conclusion: These findings extend prior studies on IgG4 in adult EoE to the pediatric population and provide deeper understanding of the potential significance and regulation of IgG4, demonstrating that IgG4 is a relevant feature of the disease; is closely related to esophageal eosinophil levels, type 2 immunity and T regulatory cytokines; and is likely produced locally.

KEYWORDS

eosinophilic esophagitis, eosinophilic oesophagitis, histology, IgG4, pediatric, transcriptome

1 | INTRODUCTION

The etiology of eosinophilic esophagitis (EoE) involves the complex interplay of genetic, host immune system, and environmental

factors.¹⁻³ A large proportion of patients with EoE are sensitized to multiple foods, but only a minority have clinical reactions consistent with IgE-mediated food allergy. Furthermore, elimination diets guided by skin prick and patch tests and serum-specific IgE levels do

not reliably identify food triggers.^{4,5} Accordingly, an anti-IgE monoclonal antibody (omalizumab) does not improve symptoms or induce histologic remission.⁶

Recent data suggest an association between elevated levels of IgG4 and EoE.⁷ Clayton et al⁶ showed that adult EoE patients had increased levels of IgG4 in esophageal tissue. Furthermore, when food triggers were eliminated, total and food-specific IgG4 levels in the esophagus decreased in adult patients who achieved histologic resolution (diet responders) but not in diet nonresponders.⁸ Though these studies provide preliminary evidence for a role of IgG4 in adult EoE, the applicability of these findings to pediatric EoE, as well the functional significance, have not been determined. Notably, high IgG4 levels are generated following allergen immunotherapy and in beekeepers, two scenarios in which individuals are exposed to high levels of antigen. IgG4 is thought to be a neutralizing antibody because it binds weakly to Fc γ receptors, does not fix complement or activate antibody-dependent cellular cytotoxicity, and undergoes Fab-arm exchange, which limits its ability to cross-link receptors and form immune complexes.⁹ It remains unknown whether alterations in IgG4 levels occur in pediatric EoE and whether IgG4 correlates with specific disease features; IgG4 may have a pathogenic role in EoE or may simply be a readout of a suppressive humoral antibody response to chronic antigen exposure.

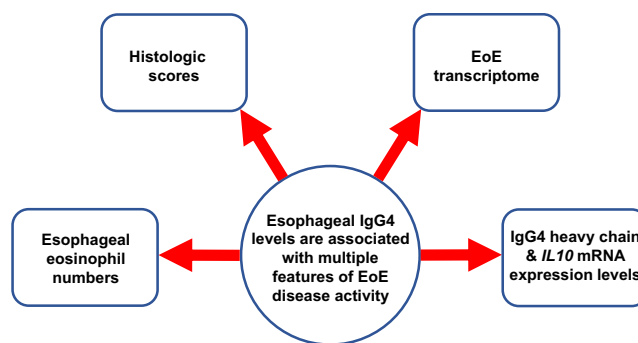
Herein, we hypothesized that (i) esophageal IgG4 levels are elevated in pediatric subjects with EoE, (ii) IgG4 levels correlate with disease activity as defined by esophageal eosinophil levels and histologic scores, (iii) IgG4 levels correlate with esophageal mRNA expression of cytokines known to influence class switching to and production of IgG4, (iv) IgG4 levels correlate with a subset of the EoE transcriptome, and (v) IgG4 is locally produced in the esophagus.

2 | MATERIALS AND METHODS

2.1 | Study design and subject selection

A case-control study was conducted with samples from pediatric subjects with EoE (N = 17) and non-EoE control subjects (N = 19). Subjects were matched for age and gender when possible and were selected without regard to atopic status. All subjects were <18 years of age and of Caucasian descent. Subjects with EoE had peak eosinophil counts of ≥ 15 eosinophils/high power field (HPF) in the distal esophagus. Subjects with EoE were selected without regard to treatment status. The control group included patients with no known history of EoE who demonstrated normal endoscopic and histologic evaluation.

Subjects who had an endoscopy for ongoing clinical care consented to provide esophageal biopsy specimens for research purposes in a study approved by the Institutional Review Board (IRB) of Cincinnati Children's Hospital Medical Center. Tissue samples obtained for research were placed in RNeasy lysis buffer (76104, Qiagen, Valencia, CA) at the time of endoscopic collection and stored at -80°C until processing.



- Esophageal IgG4 levels are increased in patients with EoE compared with control individuals and strongly correlate with esophageal eosinophil numbers and multiple features of histological grade and stage scores.
- Esophageal IgG4 protein levels correlate with multiple components of the disease as assessed by transcriptome profiling, including IL4, IL13 and IL10 mRNA expression levels.
- IgG4 heavy chain mRNA expression is proportional to IgG4 protein levels and IL10 mRNA expression levels in the esophagus of patients with EoE.

2.2 | Tissue immunoglobulin quantitation

Esophageal biopsy specimens collected in RNeasy lysis buffer were homogenized in PBS in the presence of cOmplete Protease Inhibitor Cocktail (04693132001, Roche, Mannheim, Germany) by means of the TissueLyser LT (85600, Qiagen, Germantown, MD). Supernatant protein content was measured by Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (23225, Pierce/Thermo Scientific, Rockford, IL) and adjusted to a total protein concentration of 100 μg protein/mL. IgA, IgM, and IgG subclasses (IgG1-IgG4) were measured using the Luminex 100 system (MilliporeSigma, Burlington, MA), and IgE was quantified by ELISA (25-IGE-HU-E0, ALPCO, Salem, NH). All data were normalized (milligrams immunoglobulin/grams total protein for IgA, IgM, and IgG subclasses and micrograms immunoglobulin/grams total protein for IgE).

2.3 | Histologic evaluation

Distal esophageal biopsies obtained for diagnosis were fixed in formalin, processed into paraffin blocks from which 5-micron sections were cut, and stained with hematoxylin and eosin. The slides were scored using the EoE histology scoring system (EoEHSS)¹⁰ by a pathologist (MHC) who was blinded to all subject characteristics; these biopsies were obtained from the same endoscopy as the research biopsies used for tissue immunoglobulin quantitation.

2.4 | RNA extraction and reverse transcription

Total RNA was extracted from esophageal biopsy specimens collected in RNeasy lysis buffer using the miRNeasy RNA Extraction Kit (217004, Qiagen, Germantown, MD), according to the manufacturer's protocol. Similarly, these biopsies were obtained from the same endoscopy as the research biopsies used for tissue immunoglobulin quantitation. RNA (500 ng) was reverse transcribed using ProtoScript

cDNA Synthesis Kit (E6560, New England Biolabs, Ipswich, MA), according to the manufacturer's protocol.

2.5 | Transcriptomic analysis

Transcriptomic signatures of esophageal biopsy samples were obtained using the EoE diagnostic panel (EDP) composed of a set of 96 transcripts (including one housekeeping gene, *GAPDH*).¹¹ An aliquot of cDNA equivalent to 500 ng starting RNA was adjusted to 100 μ L with H₂O and mixed with 100 μ L TaqMan Universal PCR Master Mix (4440040, Applied Biosystems, Foster City, CA) and loaded on low density qPCR fluidic cards. TaqMan real-time qPCR amplification was performed on an ABI 7900HT System (Applied Biosystems) as previously reported.¹² Interleukin-10 (*IL10*) was amplified by real-time PCR by means of SYBR Green PCR Master Mix (4309155, Applied Biosystems, Carlsbad, CA). Primers used were IL10F (5'-TGCCTTCAGCAGAGTGAAGACTT-3') and IL10R (5'-TCCTCCAGCAAGGACTCCTTTA-3'). The constant region of immunoglobulin heavy chain G4 (*IGHG4*) was amplified by TaqMan real-time PCR. Primers and probe used were IGHG4F (5'-GGACAA-GAGAGTTGAGTCCAAATA-3'), IGHG4R (5'-CATGAGAGTGTCGGG TTT-3'), and IGHG4 probe (5'-CCCATGCCCATCATGCCAG-3'). Raw cycle threshold values for each sample/each gene were exported into GraphPad Prism for statistical analysis. The expression values of the different transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Total EoE scores were calculated from the EDP as previously described.¹² Of note, the total EoE score is inversely correlated with disease activity.

2.6 | Statistical considerations

Demographic characteristics between subjects with EoE and non-EoE control subjects were compared using the Mann-Whitney *U* test for continuous variables, and Fischer's exact test was used to compare proportions between groups and to examine associations between categorical variables. To evaluate tissue immunoglobulin levels between groups, medians were compared using the Mann-Whitney *U* test. To evaluate tissue IgG4 levels among subjects with EoE on different therapies, a one-way ANOVA was performed. To determine associations between tissue IgG4 levels and histologic features, nonparametric Spearman rank correlations were calculated. To determine associations between tissue IgG4 levels and transcriptomic features, nonparametric Spearman rank correlations were calculated, and results of multiple test comparisons were corrected using the Benjamini and Hochberg false discovery rate (FDR) with FDR < .05 and adjusted *P* value < .05.

3 | RESULTS

3.1 | Subject characteristics

No significant difference in age (subjects with EoE: 7.3 years [interquartile range: 2.3-12.0]; *N* = 17) vs non-EoE controls: 8.7 years

[5.3-12.2]; *N* = 19) or gender was observed between groups; both groups had a male predominance (94% vs 79%, respectively) (Table S1). There was no significant difference in atopic status between the two groups (94% vs 74%). There was no significant difference in proton pump inhibitor use between the two groups (65% and 84%). There was no significant difference in combined atopic status and proton pump inhibitor use between the two groups (49% and 52%). In subjects with EoE, therapies for the treatment of EoE included swallowed topical steroids alone (either fluticasone propionate or budesonide), diet modification alone (elemental diet, elimination diet, and/or food trial), or both swallowed steroids and diet modification. One patient was untreated.

3.2 | Esophageal tissue immunoglobulin quantitation

Increases of IgG subclasses, as well as IgA and IgM, were seen in subjects with EoE relative to controls (Table 1). IgG1 (13.4 mg/g protein [interquartile range: 9.5-18.4] vs 9.0 mg/g protein [6.6-10.2]; *P* = .0132), IgG2 (16.2 mg/g protein [9.7-32.6] vs 8.1 mg/g protein [2.7-11.2]; *P* = .0122), IgG3 (2.2 mg/g protein [1.3-5.1] vs 0.6 mg/g protein [0.5-1.0]; *P* < .0001), IgA (2.0 mg/g protein [1.8-4.6] vs 0.9 mg/g protein [0.5-1.4]; *P* < .0001), and IgM (2.3 mg/g protein [0.9-3.9] vs 0.9 mg/g protein [0.6-1.5]; *P* = .0102) were increased in EoE compared with control individuals. IgE was not significantly different between subjects with EoE and control individuals (18.7 μ g/g protein [18.2-19.1] vs 19.5 μ g/g protein [18.8-20.4]; *P* = .0620). The greatest magnitude of change between groups was seen in IgG4 (4.2 mg/g protein [1.0-13.1] EoE vs 0.2 mg/g protein [0.1-0.9] control; *P* < .0001) (Figure 1A). A 4-fold increase in percent of IgG4 relative to total IgG was observed in EoE biopsies compared to controls (18.0% [6.5-23.5] vs 4.5% [1.8-9.3]; *P* = .0122) (Figure 1B). There were no correlations between either patient age (Figure S1A) or the duration time between EoE diagnosis and biopsy sample collection (Figure S1B) and tissue IgG4 content. There were no significant differences in tissue IgG4 levels among subjects with EoE on different therapies (*P* = .1349).

3.3 | Esophageal tissue IgG4 content and histologic features of EoE

Tissue IgG4 levels positively correlated with peak eosinophil count (Spearman ρ = .76; *P* = .0006) of a corresponding esophageal biopsy taken at the same endoscopy in subjects with EoE (Figure 2A), which was the strongest correlation of all the immunoglobulins (Table S2). Furthermore, tissue IgG4 levels correlated with mean histologic grade (ρ = .74; *P* = .0011; Figure 2B) and stage (ρ = .61; *P* = .0112; Figure 2C) scores in subjects with EoE.

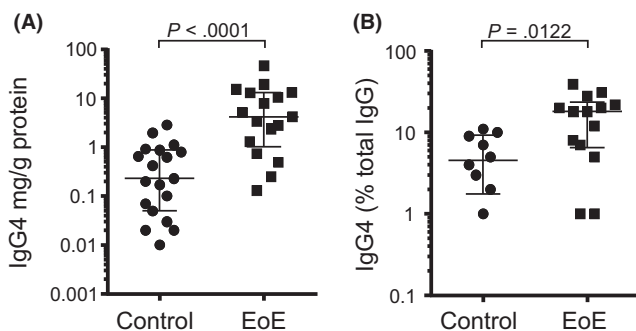
Focusing on each of the histologic features, tissue IgG4 levels correlated most substantially with the grade score of basal zone hyperplasia (ρ = .78; *P* = .0004); significant correlations were also seen with the grade scores of eosinophilic inflammation (ρ = .51; *P* = .0431) and surface epithelial alteration (ρ = .50; *P* = .0408) and

TABLE 1 Immunoglobulin content in esophageal mucosal biopsy tissue^a

Immunoglobulin, median (IQR)	Control subjects	Subjects with EoE	EoE/control ratio	P value ^b
IgE	19.5 (18.8-20.4)	18.7 (18.2-19.1)	1.0	.0620
IgA	0.9 (0.5-1.4)	2.0 (1.8-4.6)	2.2	<.0001
IgM	0.9 (0.6-1.5)	2.3 (0.9-3.9)	2.6	.0102
IgG1	9.0 (6.6-10.2)	13.4 (9.5-18.4)	1.5	.0132
IgG2	8.1 (2.7-11.2)	16.2 (9.7-32.6)	2.0	.0122
IgG3	0.6 (0.5-1.0)	2.2 (1.3-5.1)	3.7	<.0001
IgG4	0.2 (0.1-0.9)	4.2 (1.0-13.1)	21.0	<.0001
IgG4, % total IgG	4.5% (1.8-9.3)	18.0% (6.5-23.5)	4.0	.0122

^aIgE measurements are reported in micrograms immunoglobulin per gram of total protein. IgA, IgM, and IgG1-IgG4 measurements are reported in milligrams immunoglobulin per gram of total protein.

^bComparison of medians by Mann-Whitney U test.

**FIGURE 1** Esophageal tissue IgG4 content. The data are expressed as milligrams of IgG4 per gram of total protein (A) and as a fraction of total IgG (B). Each data point represents the mean IgG4 measurement from an individual distal biopsy from 17 biologically independent subjects. The lines represent the median and interquartile range for each group

the stage scores of basal zone hyperplasia ($\rho = .64$; $P = .0070$), eosinophilic inflammation ($\rho = .49$; $P = .0468$), eosinophil surface layering ($\rho = .50$; $P = .0448$), dilated intercellular spaces ($\rho = -.49$; $P = .0490$), and surface epithelial alteration ($\rho = .49$; $P = .0479$) (Figure 2D). Biopsies of 11 out of 17 subjects with EoE included lamina propria; in this subset, tissue IgG4 levels did not correlate with the grade or stage scores of lamina propria fibrosis (respectively, $\rho = .22$; $P = .5160$ and $\rho = .23$; $P = .5368$).

In non-EoE control subjects, there were no significant correlations between tissue IgG4 levels and peak eosinophil count ($\rho = -.07$; $P = .7612$), mean histologic grade score ($\rho = -.29$; $P = .2474$), or mean histologic stage score ($\rho = -.07$; $P = .7754$).

3.4 | Esophageal tissue IgG4 content and cytokine mRNA expression in the esophageal mucosa

Tissue IgG4 protein levels correlated with esophageal *IL4* ($\rho = .51$; $P = .0366$) (Figure 3A), *IL13* ($\rho = 0.67$; $P = .0040$) (Figure 3B), and *IL10* ($\rho = .49$; $P = .0465$) (Figure 3C) mRNA expression levels in subjects with EoE. In contrast, there was no correlation between tissue

IgG4 levels and transforming growth factor beta (*TGFB1*) mRNA expression levels (Figure 3D).

3.5 | Expression of IgG4 heavy chain mRNA in the esophageal mucosa

A large variation in the *IGHG4* mRNA levels was observed in esophageal biopsies. However, *IGHG4* mRNA was present in the esophageal mucosa and was increased in subjects with EoE compared with control individuals ($P = .0413$) (Figure S2). Representative *IGHG4* PCR products were sequenced to confirm their identities compared to the corresponding GenBank *IGHG4* sequence (AJ294733.1).

Tissue IgG4 levels correlated with esophageal *IGHG4* mRNA expression levels ($\rho = .56$; $P = .0203$) in subjects with EoE (Figure 4A). Esophageal *IGHG4* mRNA expression levels correlated with *IL10* mRNA expression levels ($\rho = .57$; $P = .0191$) (Figure 4B), but not *IL4*, *IL13*, and *TGFB1* mRNA expression levels in subjects with EoE (Figure S3A-C). In non-EoE control individuals, esophageal *IGHG4* mRNA expression levels did not correlate with *IL10* mRNA expression levels (Figure S4).

3.6 | Esophageal tissue IgG4 content and a diagnostic subset of the EoE transcriptome

Tissue IgG4 levels correlated with total scores from the EoE Diagnostic Panel, a set of 96 genes expressed in the esophagus ($\rho = -.71$; $P = .0021$) (Figure S5).¹² Nonparametric correlations identified 62 genes with expression levels that significantly correlated with tissue IgG4 levels (following false discovery rate-corrected $P < .05$). There was a diverse set of functional categories among these 62 genes, including cell adhesion (4 of 5 genes), chemokines and cytokines (5 of 6 genes), epithelial barrier (11 of 12 genes), inflammation (16 of 22 genes), ion channels (4 of 4 genes), and proliferation (6 of 7 genes) (Figure 5). Of note, levels of mast cell-specific transcripts (eg, *CPA3*) did not correlate with IgG4 levels ($P > .0647$).

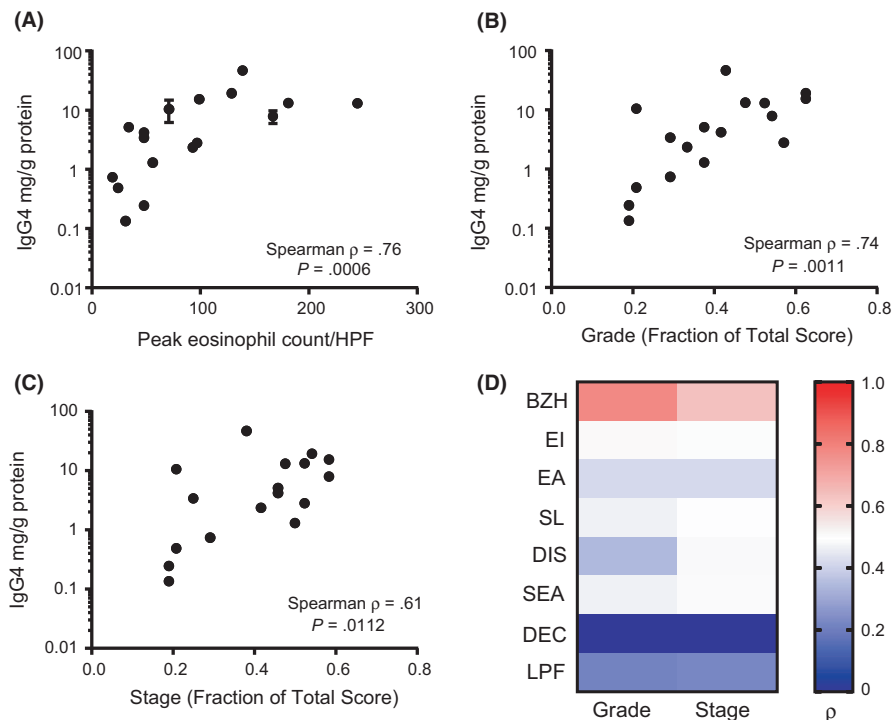


FIGURE 2 Esophageal tissue IgG4 content and histologic features of eosinophilic esophagitis (EoE). A, Correlation between esophageal peak eosinophil count (peak eosinophil count/HPF) and esophageal tissue homogenate IgG4 content (mg IgG4 per gram of total protein) in subjects with EoE. Each data point represents the mean IgG4 measurement from an individual distal biopsy from 17 biologically independent subjects. The lines represent the standard deviation for each data point. B, Correlation between histologic grade (as a fraction of the total histologic score) and esophageal tissue homogenate IgG4 content (mg IgG4 per gram of total protein) in subjects with EoE. C, Correlation between histologic stage (as a fraction of the total histologic score) and esophageal tissue homogenate IgG4 content (mg IgG4 per gram of total protein) in subjects with EoE. D, A heat map was generated demonstrating the degree of correlation between esophageal tissue IgG4 content and each of the histologic features by grade and stage. Red indicates a higher degree of correlation (closer to Spearman ρ of 1.0) and blue represents a lower degree of correlation (closer to a Spearman ρ of zero). The magnitude of the color change is proportional to the strength of the correlation. Eosinophil inflammation (EI) was evaluated using peak eosinophil counts obtained by counting eosinophils in the most densely inflamed HPF. Additional features were basal zone hyperplasia (BZH): >15% of the total epithelial thickness; eosinophil abscess (EA): solid mass of intraepithelial eosinophils; eosinophil surface layering (SL): linear alignment of eosinophils parallel to the epithelial surface; dilated intercellular spaces (DIS): spaces around squamous epithelial cells that exhibit intercellular bridges; surface epithelial alteration (SEA): surface epithelial cells that exhibit altered tinctorial properties, manifest as dark red staining, with or without intraepithelial eosinophils; dyskeratotic epithelial cells (DEC): individual cells with deeply eosinophilic cytoplasm and hyperchromatic nuclei; lamina propria fibrosis (LPF): thickened connective tissue fibers in the lamina propria

3.7 | Detection of IgG4 in the esophageal mucosa

IgG4 immunofluorescent staining of esophageal mucosal biopsy specimens showed diffuse intercellular staining in the epithelial compartment of 6 of 8 subjects with EoE compared to 0 of 6 normal controls ($P = .0097$) (Figure S6). Rare IgG4-positive plasma cells, all of which stained for CD319, were seen in the lamina propria in 3 of 8 subjects with EoE and in the epithelial compartment of 1 of 8 subjects with EoE (Figure S7). All control tissue specimens lacked IgG4-positive plasma cells.

4 | DISCUSSION

This study is the first to evaluate esophageal immunoglobulin levels in the pediatric EoE population. We report the following findings: (i)

there is a diffuse increase in multiple immunoglobulin (Ig) isotypes in the esophagus of patients with EoE compared with control individuals, including IgA, IgM, and all IgG subclasses, but not IgE; (ii) of all Ig isotypes, IgG4 shows the largest increase (ie, a 21-fold change); and (iii) increases of IgG4 occur independent of age and duration of disease. Additionally, these data demonstrate: (i) levels of esophageal IgG4 protein correlate with the cardinal feature of the disease, esophageal eosinophil numbers, indicating that IgG4 is reflective of disease activity; (ii) there is a strong correlation between IgG4 levels and multiple features of histologic grade and stage scores with the strongest association seen with basal zone hyperplasia and no association with lamina propria fibrosis (albeit, sample size may be a limiting factor); (iii) mechanistically, IgG4 protein levels correlated with levels of *IL4*, *IL13*, and *IL10* but not *TGFB1* mRNA; (iv) IgG4 heavy chain mRNA expression is increased and proportional to IgG4 protein levels and *IL10* mRNA expression levels in the esophagus; and

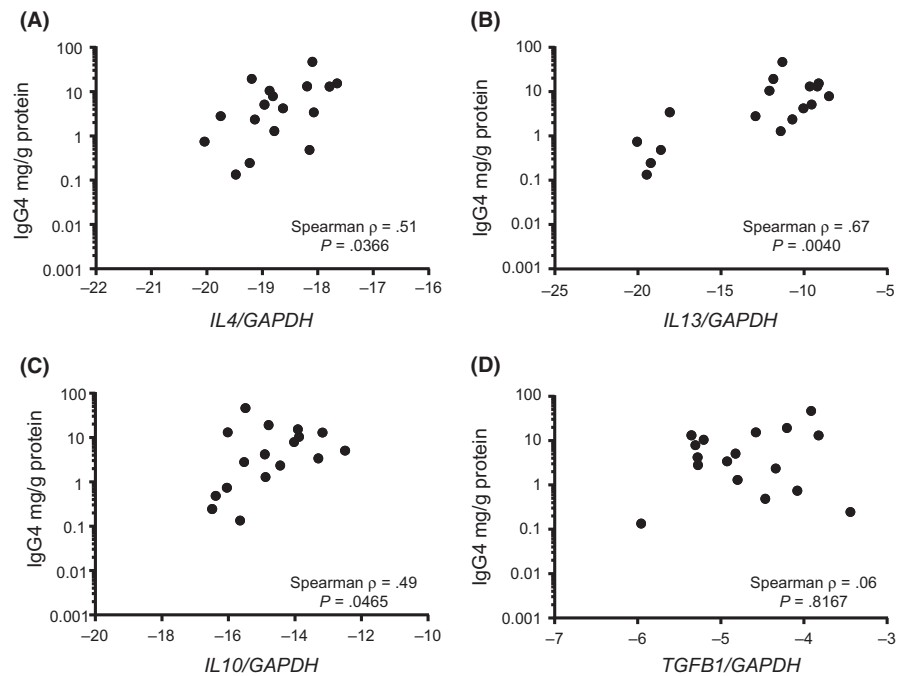


FIGURE 3 Esophageal tissue IgG4 content and cytokine mRNA expression in the esophageal mucosa. Correlations between mRNA expression of (A) *IL4*, (B) *IL13*, (C) *IL10*, and (D) *TGFB1* and esophageal IgG4 content (mg IgG4 per gram of total protein) in esophageal biopsies from subjects with eosinophilic esophagitis

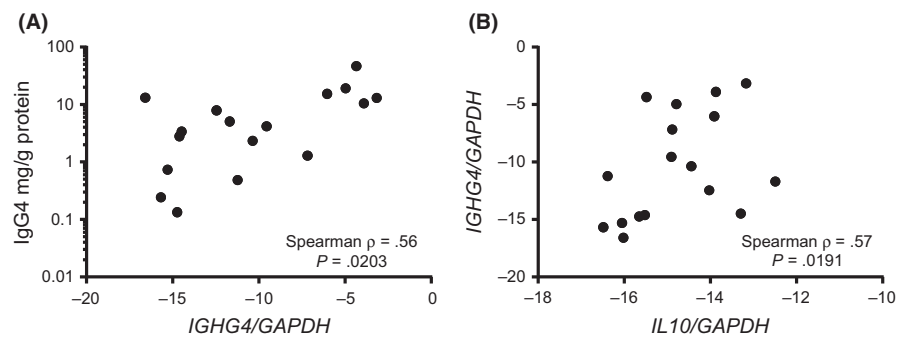


FIGURE 4 Expression of IgG4 heavy chain mRNA in the esophageal mucosa. A, Correlation between *IGHG4* mRNA expression and IgG4 protein content (mg IgG4 per gram of total protein) in esophageal biopsies from subjects with eosinophilic esophagitis (EoE). B, Correlation between *IL10* mRNA expression and *IGHG4* mRNA expression in esophageal biopsies from subjects with EoE

(v) esophageal IgG4 protein levels strongly correlate with multiple components of the disease as assessed by transcriptome profiling, although no correlation was seen with transcript markers of mast cell tissue burden.¹³ Collectively, these findings extend prior studies focused on IgG4 in adult EoE to the pediatric population and provide deeper understanding of the potential significance and regulation of IgG4. In particular, these data show that IgG4 is a relevant feature of the disease rather than an epiphenomenon associated with a subset of patients, is closely related to esophageal eosinophil counts and degree of basal zone hyperplasia, and is likely produced locally, at least in part.

Whereas increases in IgA, IgM, and the other IgG subclasses were detected, the greatest change observed was in IgG4. Of all the immunoglobulins, IgG4 had the strongest correlation with esophageal eosinophil numbers. These data suggest that the local immunoglobulin response in EoE is predominantly IgG4. Tissue IgE levels were not different between subjects with EoE and controls, which prompts consideration that the esophageal cytokine environment in EoE may shift toward isotype switching to IgG4 and not IgE. T helper cell type 2 (Th2) cytokines IL-4 and IL-13 are responsible for IgG4 and IgE production by B cells.¹⁴ In allergic immune responses,

IgG4 production and preferential isotype switching to IgG4 rather than IgE are induced by the activation of IL-10 produced by regulatory T (Treg) cells.^{7,15-19} In view of these findings and previous work characterizing Th2 cytokines^{20,21} and T regulatory cells in EoE,^{22,23} including a study demonstrating local immunoglobulin class switching to IgE and IgE production in the esophageal mucosa of EoE patients,²⁴ we sought to provide evidence of local IgG4 production. Indeed, these data demonstrate that local *IGHG4* mRNA expression is a feature of the esophageal mucosa of EoE. Furthermore, the correlations of tissue IgG4 levels with esophageal *IL10*, *IL13*, and *IL4* mRNA expression suggest that a combined Th2 and Treg environment exists locally, which likely supports class switching to and production of IgG4 in the esophageal mucosa. The role of *IL13* in EoE pathogenesis has previously been well characterized,^{20,25,26} and differential *IL10* mRNA expression between EoE and control subjects has been demonstrated in other studies²⁷ and by novel reanalysis of RNA sequencing data (0.14 RPKM [interquartile range: 0.11-0.21] vs 0.02 RPKM [0.01-0.10]; $P < .0001$).^{28,29} Notably, tissue IgG4 levels did not correlate with *TGFB1* mRNA esophageal expression. Though previous studies have shown that TGF- β cooperates with IL-10 in the normal mucosal response to antigens,³⁰ TGF- β can also participate in pro-

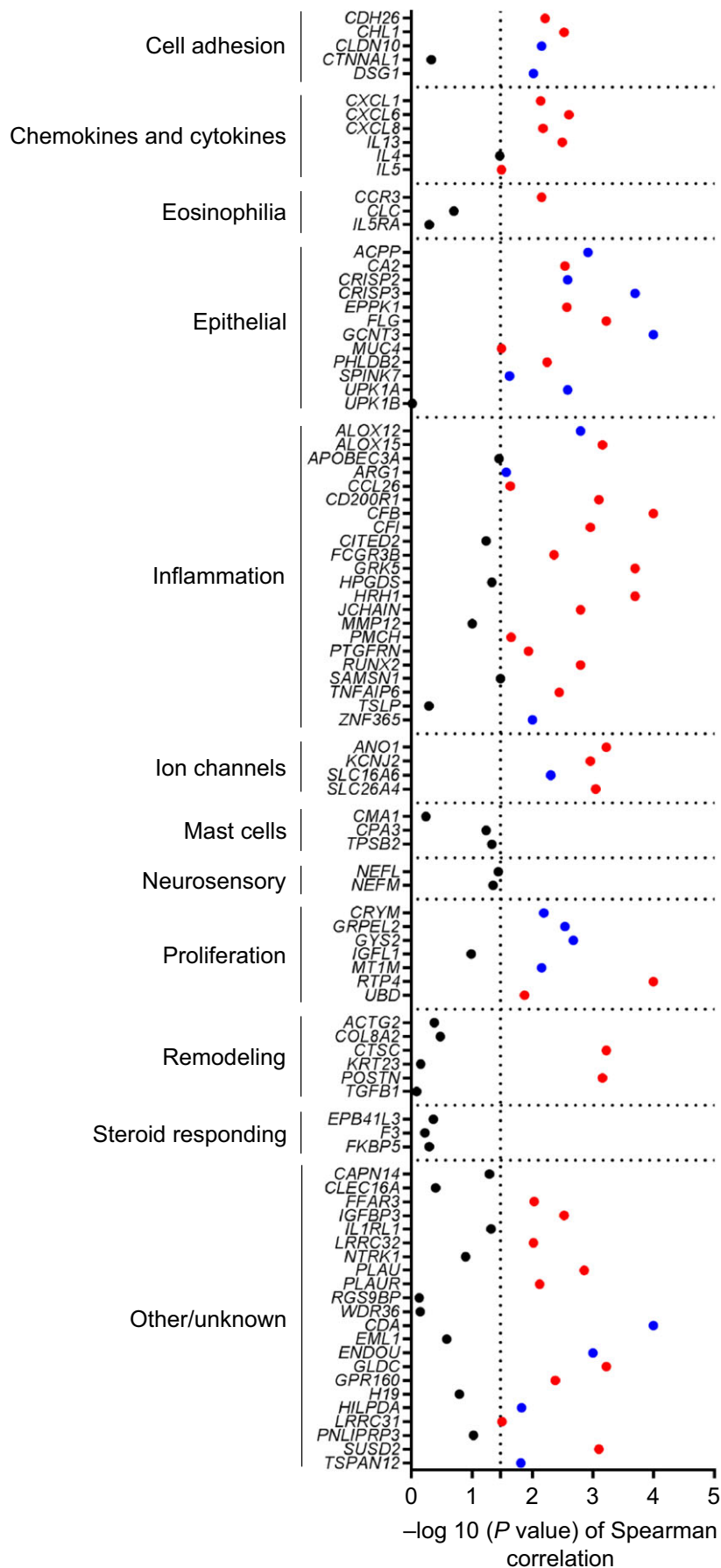


FIGURE 5 Associations between esophageal tissue IgG4 content and a diagnostic subset of the eosinophilic esophagitis (EoE) transcriptome. The x-axis represents the negative log₁₀ P value of the Spearman correlation between tissue IgG4 levels and a diagnostic subset of genes from the EoE transcriptome (EDP). Genes shown on the y-axis are organized within functional groupings. Red shading denotes upregulated genes, and blue shading denotes downregulated genes. The vertical dashed line indicates FDR-corrected $P < .05$, and the horizontal dashed lines denote separation of the functional groupings of genes.

inflammatory responses³¹; in fact, eosinophil-derived TGF- β is linked with epithelial growth, fibrosis, and tissue remodeling.²¹ Thus, a correlation between tissue IgG4 levels and *TGFB1* expression may not have been detected as TGF- β is a pleiotropic cytokine secreted by many cell types including eosinophils, T cells, and mast cells.

The downstream effects of Th2 cytokines include priming of naïve B cells to produce IgG4. Excess pro-inflammatory Th2 responses, as seen in clinical settings involving chronic antigen exposure (such as in beekeepers and during allergen immunotherapy), are known to induce Treg cells, which secrete high levels of IL-10, inducing class switching to IgG4. In addition to Treg cells, B regulatory cells and eosinophils are potential sources of IL-10,^{32–34} although it remains to be determined whether they are local producers of IL-10 in EoE. IgG4 production may be a compensatory mechanism in an effort to dampen the ongoing type 2 inflammatory responses in EoE. The associations between tissue IgG4 levels and the EDP categories of inflammation, signaling molecules, epithelium, and cell adhesion support that IgG4 production is indeed associated with cardinal features of the type 2 immune response associated with this disease. It is notable that no correlation was seen between IgG4 levels and markers of mast cell burden (eg, *CPA3*), suggesting that mast cells are not the primary cell driving IgG4 production (ie, mast cells are not the major producers of IgG4 class switching regulating molecules). A potential role of IgG4 is as a “blocking antibody” binding food antigen before it encounters IgE bound to the surface of mast cells or IgG4-dependent activation of inhibitory Fc γ receptors.^{17,35} Also, the emergence of IgG4 and decreasing levels of IgE can be predictive of tolerance to antigen and predictive of success in oral immunotherapy.³⁶ The close association of IgG4, EoE, and IgE is supported by the observation that a substantial number of patients undergoing oral immunotherapy for IgE-mediated food allergy subsequently develop EoE.³⁷ It is plausible that at least a subset of EoE patients may have an IgE-associated pathology (especially initially), but with repeated exposure to culprit food antigens, the imbalanced production of IgG4 over IgE may predominate, as acute reactions are not seen, and thus, antigen exposure continues and then EoE exacerbates. In support of this, previous studies have observed that IgE antibodies are ultimately not involved in sustaining disease activity.^{1,6,38,39} Overall, it is interesting to speculate that IgG4 distinguishes EoE from classic food anaphylaxis as IgG4 may promote permissive food ingestion (blocking anaphylaxis), which promotes the Th2-driven pathology characteristic of EoE.

In line with recent evidence in the literature,^{6,40,41} the presence of IgG4 immunostaining in the esophageal epithelium strengthens the association of IgG4 and EoE. Though previous studies have also reported IgG4 staining in the lamina propria, including IgG4-positive plasma cells, a similar analysis was limited by the small sample size with sufficient lamina propria. Focusing on histologic features, basal zone hyperplasia was noted to have the strongest correlation with tissue IgG4 levels. Though basal zone hyperplasia is also a typical feature of other pathologies, for example, GERD, this correlation highlights the fact that tissue IgG4 levels are associated with

multiple features of disease activity as basal zone hyperplasia is a pathologic feature not defined by eosinophils.¹⁰ Though there are multiple causes of basal zone hyperplasia, the causes in EoE and GERD appear to be different; for example, IL-13, which is pathogenic in EoE, leads to epithelial hyperplasia.²⁶ Conversely, no correlation was found between tissue IgG4 levels and lamina propria fibrosis; however, this analysis may have been limited by the small number of biopsy samples that included lamina propria. Fibrosis is a characteristic feature of both IgG4-related disease (IgG4-RD) and the fibrostenotic phenotype of EoE, although the quality of fibrosis is generally dissimilar; IgG4-RD is typified by storiform-type fibrosis,⁴² whereas lamina propria fibrosis in EoE demonstrates no specific pattern. Though there are overlapping features between the two diseases in that both demonstrate a response to corticosteroid treatment and are associated with atopy, tissue eosinophilia, fibrosis, and IgG4-positive plasma cells,^{6,42} the predominant features of the two diseases are different. Dense lymphoplasmacytic infiltrate, storiform fibrosis, and obliterative phlebitis are characteristic of IgG4-RD, whereas eosinophilic inflammation is the distinguishing histopathologic finding in EoE. Furthermore, the esophagus is rarely involved in IgG4-RD.^{43–47} Currently, EoE is not considered to be on the spectrum of IgG4-RD; however, IgG4-RD may serve as a paradigm to investigate the biological basis of IgG4 and its involvement in EoE. For example, transcriptomic analysis in patients with IgG4-RD revealed differences in mRNA levels of IgG4 among patients with different clinical manifestations of IgG4-RD, and differences in immune and inflammatory cell gene signatures correlated with disease activity.⁴⁸

The limitations of this study include its retrospective and cross-sectional design that did not allow for longitudinal investigation of tissue IgG4 levels. Furthermore, this study was not powered to investigate differences between patients with treatment-naïve EoE and those on therapy as it is possible that therapies to treat EoE, for example, swallowed steroids and/or diet modification, affect IgG4 production and other immunoglobulins. It will be important for future studies to investigate changes in the EoE transcriptome relative to tissue IgG4 levels in patients with active and inactive disease. Similarly, future studies with larger sample sizes are necessary to explore the effects of atopic status and proton pump inhibitor use on tissue IgG4 levels. Prospective and interventional studies will help determine the clinical relevance of IgG4 as a biomarker, for example, its specificity to EoE. In support of this, previous studies have suggested that extracellular IgG4 deposits distinguish EoE from GERD.⁴⁰ Serologic IgG4 studies may help clarify whether food-specific serum IgG4 levels may be a noninvasive method for the identification of offending foods. The mechanistic basis for the association of IgG4 with EoE is still enigmatic and is a question that future studies must address.

In summary, esophageal IgG4 levels are elevated in pediatric EoE and correlate with disease activity as assessed by esophageal eosinophil counts, histologic scores, and transcriptomic features of EoE, providing evidence that tissue IgG4 level is a biomarker of disease activity, is closely related to type 2 immunity and T regulatory cytokines, especially *IL10*, and is likely produced locally.

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CONFLICTS OF INTEREST

PCF has served as a consultant for Genentech and has received research funding from Knopp Biosciences. VAM reports grants and personal fees from Shire. PEP has received payment for lectures from Abbott Nutrition. TW has a patent on the EoE diagnostic panel that is owned by Cincinnati Children's Health Medical Center. MER is a consultant for PulmOne, Spoon Guru, Celgene, Shire, AstraZeneca, GlaxoSmithKline, Allakos, Adare, Regeneron, and Novartis; has an equity interest in the first three listed and Immune Pharmaceuticals; and receives royalties from reslizumab (Teva Pharmaceuticals) and UpToDate. MER is an inventor of patents owned by Cincinnati Children's Hospital Medical Center. The rest of the authors declare that they have no relevant conflict of interests.

AUTHOR CONTRIBUTIONS

MER conceived the project and supervised the study. CER and MKM contributed to study design. CER and MKM acquired, analyzed, and interpreted the data with assistance from DWM, JMC, MHC, PCF, VAM, PEP, TS, and TW. CER wrote the initial draft of the manuscript. All of the authors participated in revision of the manuscript and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Esophageal IgG4 Levels Correlate with Histopathologic and Transcriptomic Features in Eosinophilic Esophagitis

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Supplementary Methods

Immunofluorescence

Formalin-fixed, paraffin-embedded esophageal tissues were selected from a separate cohort of 8 subjects with EoE and 6 non-EoE control subjects. Deparaffinization was performed using xylene-ethanol. Antigen retrieval was done in a pressure cooker at 125°C for 30 seconds in sodium citrate buffer (pH 6.0), and then allowed to cool for 15 more minutes. Ten percent donkey serum (D9663-10ML, Sigma-Aldrich, St. Louis, MO) was used to block nonspecific binding. Tissues were exposed to anti-human primary antibodies overnight at indicated concentrations: rabbit IgG4 (1.5 ug/mL, AB109493, Abcam, Cambridge, MA) and goat CD319 (8.0 ug/mL, AF1906-SP, R&D Systems, Minneapolis, MN). Following washes, secondary donkey antibodies anti-rabbit Alexa Fluor 647 and donkey anti-goat Alexa Fluor 488 (3.0 ug/mL, Jackson ImmunoResearch, WestGrove, PA) were applied. Slides were washed in phosphate-buffered saline (PBS) containing 4',6-diamidine-2'-phenylindole dihydrochloride (1.0 ug/mL DAPI; 15 minutes), cover-slipped using antifade medium (Prolong Gold, Life Technologies, Eugene, OR), and visualized on a Nikon Eclipse Ti-E outfitted with a Nikon A1R confocal scan head. Human tonsil biopsy sections were used as positive control tissue. Negative controls included unstained esophageal tissues from patients with EoE and non-EoE control individuals, controls with normal rabbit and goat sera, and secondary antibody only. All samples were immunostained under identical conditions and imaged with identical microscope settings.

Supplementary Table 1. Characteristics of the study cohort

Characteristics	Subjects with EoE (N = 17)	Control subjects (N = 19)	P value ^a
Mean age $\pm$ SD (median [IQR]) (years)	9.1 $\pm$ 4.8 (7.3 [2.3-12.0])	7.4 $\pm$ 4.8 (8.7 [5.3-12.2])	0.3751
Gender (% male)	16 (94%)	15 (79%)	0.1886
History of atopy	16 (94%)	14 (74%)	0.1821
Mean peak eosinophil count $\pm$ SD (median [IQR]) (eosinophils/HPF)	90.0 $\pm$ 63.4 (71 [41-134])	0.2 $\pm$ 0.5 (0)	< 0.0001
Treatment with PPI ^b	11 (65%)	16 (84%)	0.2553
Diet alone ^b	12 (70%)	N/A	N/A
Swallowed steroids alone ^b	2 (12%)	N/A	N/A
Diet and swallowed steroids ^b	2 (12%)	N/A	N/A
Untreated ^b	1 (6.0%)	N/A	N/A

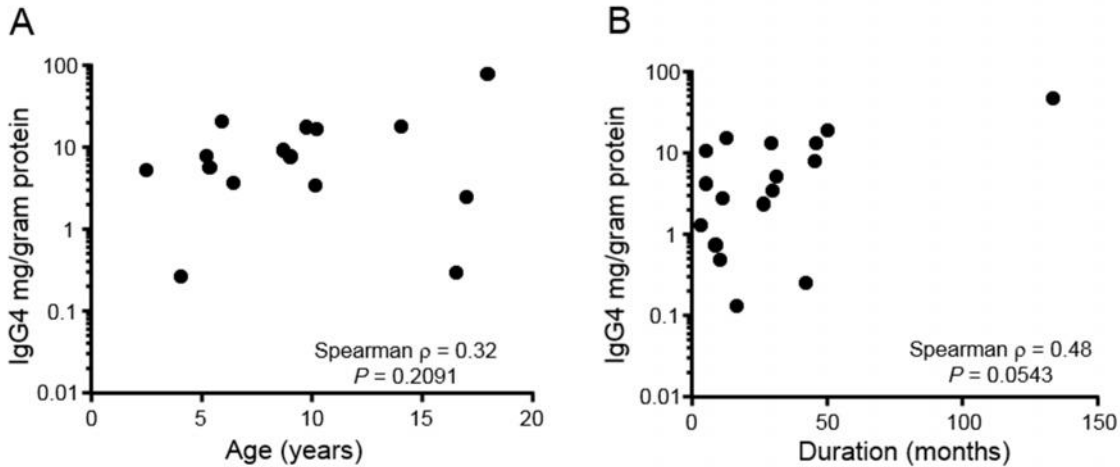
IQR – interquartile range.

^aComparison of medians by Mann-Whitney U test for age and peak eosinophil count and comparison of proportions by Fischer's exact test for gender, atopic status, and PPI use.

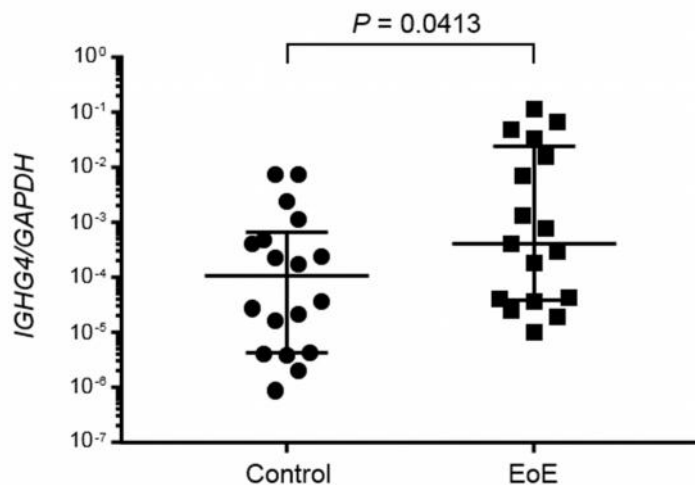
^bTreatment at the time of biopsy sample collection.

Supplementary Table 2. Correlation values between esophageal peak eosinophil count and esophageal tissue immunoglobulin content in subjects with EoE.

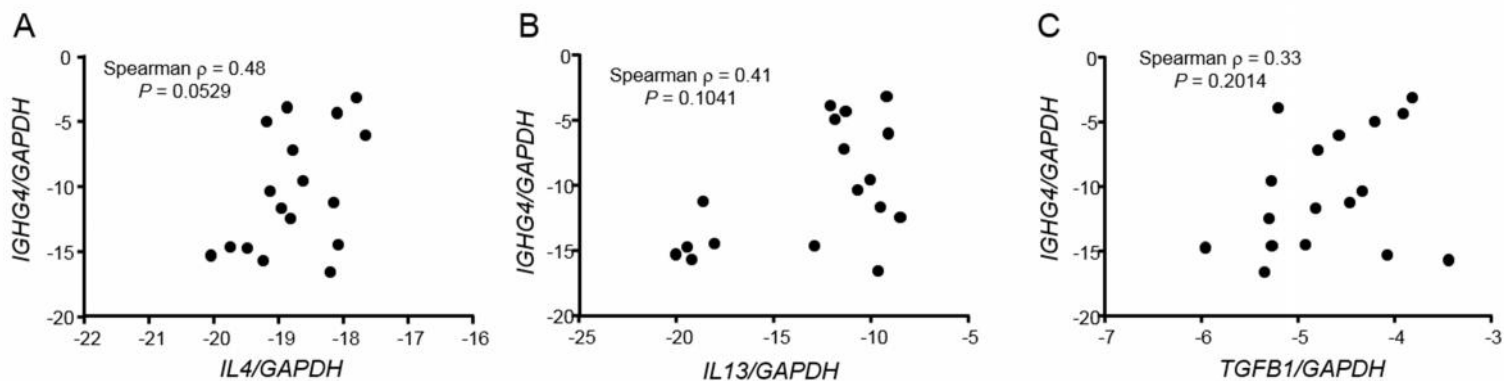
Immunoglobulin	Spearman ρ	P value
IgE	-0.06	0.8291
IgA	0.59	0.0139
IgM	0.62	0.0095
IgG1	0.69	0.0042
IgG2	0.33	0.2476
IgG3	0.60	0.0121
IgG4	0.76	0.0006



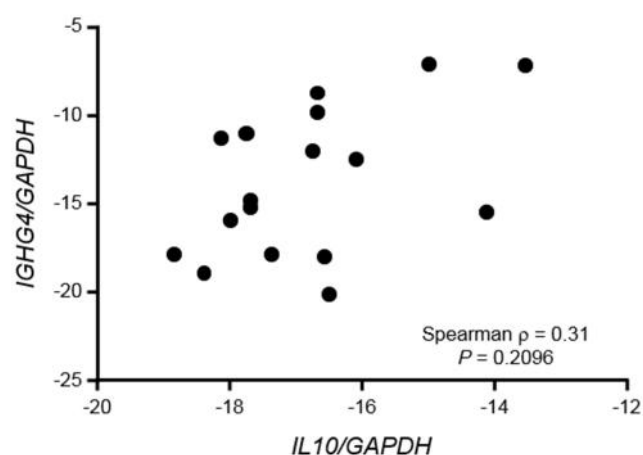
Supplementary Figure 1. Esophageal tissue IgG4 content and age and duration of disease. (A) Correlation between patient age (in years) at time of biopsy sample collection and esophageal tissue IgG4 content (mg IgG4 per gram of total protein). (B) Correlation between the duration of time between EoE diagnosis and time of biopsy sample collection (in months) and esophageal tissue IgG4 content (mg IgG4 per gram of total protein).



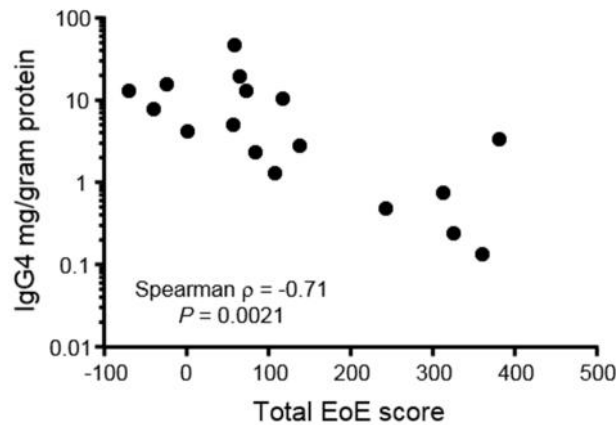
Supplementary Figure 2. Expression of IgG4 heavy chain mRNA in esophageal biopsies from EoE and control subjects. Each data point represents the mean *IGHG4* mRNA value normalized to *GAPDH* mRNA expression from the same cDNA. The lines represent the median and interquartile range for each group.



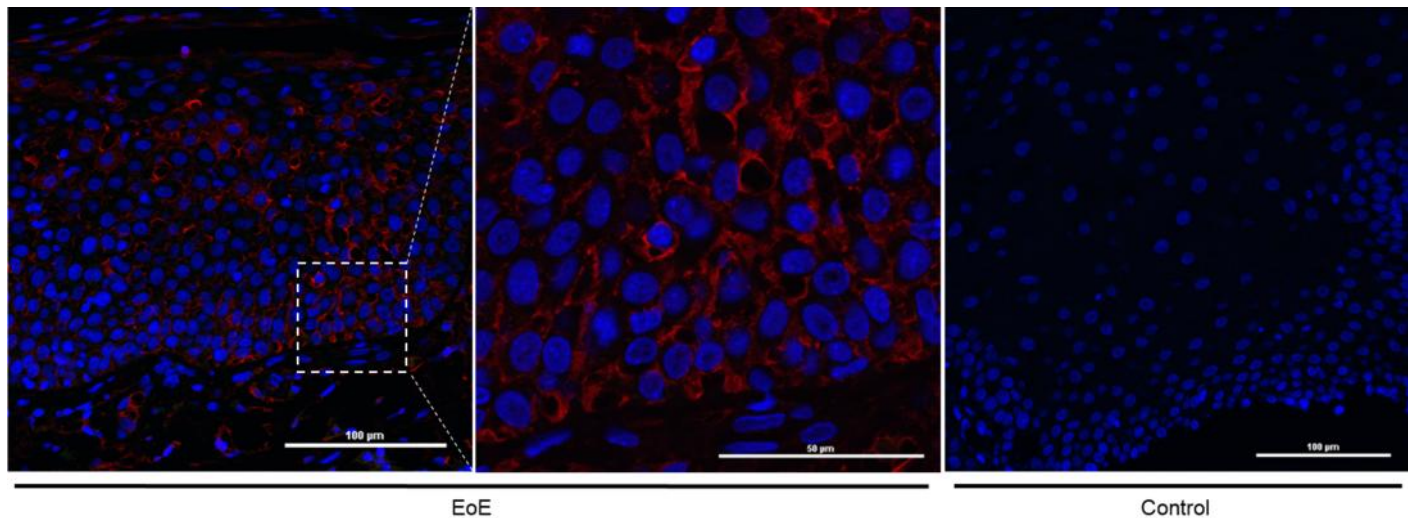
Supplementary Figure 3. Esophageal IgG4 heavy chain mRNA and cytokine mRNA expression in the esophageal mucosa of subjects with EoE. Correlations between mRNA expression of (A) *IL4*, (B) *IL13*, and (C) *TGFB1* and *IGKG4* mRNA expression in esophageal biopsies from subjects with EoE.



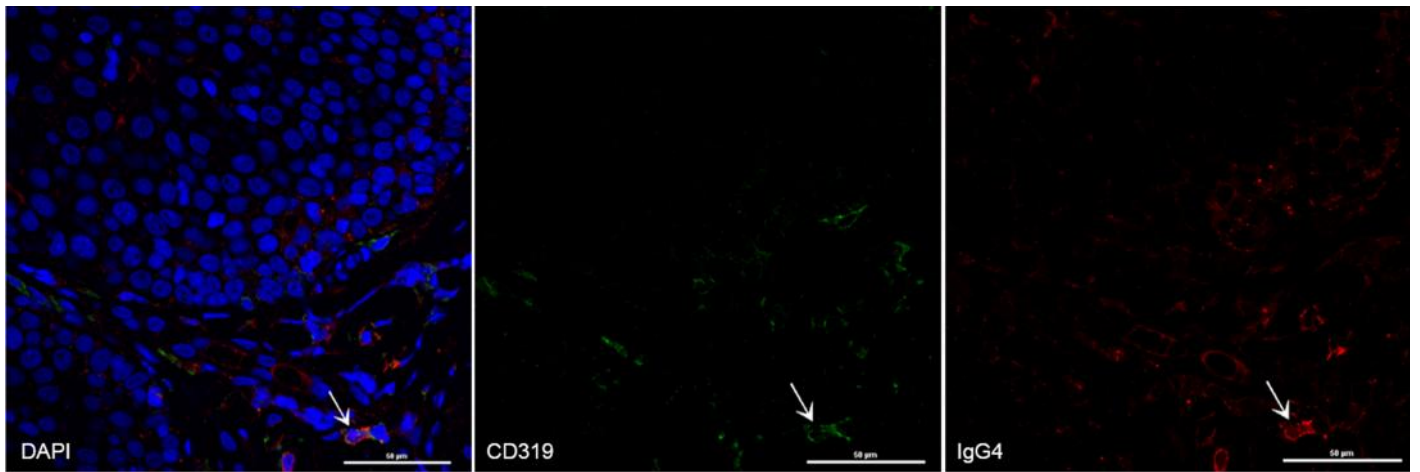
Supplementary Figure 4. Correlation between *IL10* and *IGKG4* mRNA expression levels in esophageal biopsies from non-EoE control subjects.



Supplementary Figure 5. Esophageal tissue IgG4 content and a diagnostic subset of the EoE transcriptome. Correlation between total EoE scores and esophageal tissue IgG4 content (mg IgG4 per gram of total protein) in subjects with EoE. To establish a molecular EoE diagnosis, the total EoE score was calculated from the EDP as previously described (12). Of note, the EoE score is inversely correlated with disease activity (as defined by esophageal eosinophil count).



Supplementary Figure 6. Detection of IgG4 in the esophageal mucosa. Immunofluorescent staining for IgG4 in an esophageal mucosal biopsy from a subject with EoE shows diffuse intercellular IgG4 staining, in red, with a blue DAPI nuclear counterstain. In this esophageal biopsy, the IgG4 staining is present in the epithelial compartment and the lamina propria. The control subject demonstrates no IgG4 staining.



Supplementary Figure 7. There is a single IgG4-positive plasma cell in the subepithelial lamina propria (arrow) in an esophageal mucosal biopsy from a subject with EoE. Nuclei are blue (DAPI), CD319 is green (AF488), and IgG4 is red (AF647).