IgG4 is Elevated in Eosinophilic Esophagitis but Not in Gastroesophageal Reflux Disease Patients

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Background: For eosinophilic esophagitis (EoE) recently an association with immunoglobulin (Ig)G4 rather than IgE has been reported. Gastroesophageal reflux disease (GERD) is the most important differential diagnosis of EoE. We compared esophageal IgG4 plasma cell infiltration and serum IgG4 levels of EoE patients (before and after budesonide therapy) with GERD patients.

Methods: Prospectively collected serum samples of 17 EoE patients before and after 8 weeks of therapy with budesonide (1 mg BID) were analyzed for total and antigen-specific IgG4 and IgE levels. Also, immunohistochemical analysis of total and IgG4-positive plasma cells was performed on esophageal biopsies of these patients. In total, 14 GERD patients without histologic proof of eosinophilic infiltration were taken as a control group.

Results: Total IgG4 serum levels in EoE patients were significantly higher than in GERD patients (121.0 vs. 71.2 mg/dL; $P = 0.038$) and decreased under budesonide therapy (121.0 vs. 104.2 mg/dL; $P = 0.019$). IgE levels did not differ significantly between all groups. In EoE patients also a high number of esophageal IgG4-positive plasma cells was detected and significantly reduced under therapy (29.1 vs. 0.1 IgG4-positive cells; $P < 0.001$). In GERD patients no relevant esophageal plasma cell infiltration could be seen.

Conclusions: In EoE patients elevated systemic IgG4 serum levels compared with GERD patients can be seen and decrease under topical steroid therapy. Also, local IgG4 plasma cells expression is high in EoE, but not in GERD patients and normalize under therapy. These findings are further proof for a possible association of EoE with IgG4.

Key Words: eosinophilic esophagitis, gastroesophageal reflux disease, EoE, GERD, inflammation, IgG4

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Eosinophilic Esophagitis (EoE) is a chronic immune disease of the esophagus, which is the second most prevalent disease of the esophagus after gastroesophageal reflux disease (GERD), its most important differential diagnosis.[1] The prevalence of EoE is estimated 44 to 56 per 100,000 persons in the United States and Europe.[2,3] Atopic disorders such as asthma, eczema, and food allergy are increased in EoE patients.[4] Until recently, EoE was seen as an immunoglobulin (Ig)E-mediated disease. However, this concept has been doubted and recently an association with IgG4, but not with IgE has been reported.[5]

The aim of this study was to investigate the expression of IgG4 and IgE in patients with active EoE before and after 8 weeks of topic therapy with budesonide. Total and food-specific IgG4 and IgE levels were measured before and after therapy. Also, the plasma cell infiltration and IgG4 expression in the esophageal mucosa was investigated using immunohistochemistry (IHC). The results were compared with a cohort of patients with therapy-naive GERD.

METHODS

Study Design and Study Population

This study was performed as a secondary analysis of the HIMEOS study of Nennstiel et al[6] in which high-resolution manometry data of EoE patients were evaluated before and after topical steroid therapy. The original study was approved by the local ethic committee and registered at ClinicalTrials.gov (Identifier: NCT02331849). Consecutive patients between 21 and 66 years of age with a confirmed clinicopathologic diagnosis of active EoE were eligible: clinical symptoms of esophageal dysfunction (based on a dysphagia score ≥ 3 as published before,[7] measuring frequency of dysphagia ranging from none (0 points) to several times a day (4 points) as well as intensity of dysphagia (0 to 5 points), thus ranging from 0 to 9 points in the total score) and peak eosinophils (eos) ≥ 15 eos/hpf (high power field) in at least 1 hpf derived from 6 biopsies, 2 each from the proximal, mid, and distal esophagus. All these patients received treatment with topical budesonide 2×1 mg/d for a period of 8 weeks. Patients were neither on diet before nor during therapy. GERD patients were diagnosed by typical clinical reflux symptoms (such as acid regurgitation and heartburn) and either endoscopic signs of erosive refluxesophagitis or positive ph-metry. All GERD patients had no endoscopic findings suggestive for EoE and histologically no eosinophilia in the esophageal mucosa. Written informed consent was collected from all patients. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Endoscopy

Upper endoscopy was performed by board-certified gastroenterologists at baseline and at the end of treatment. Endoscopic findings were recorded due to characteristic abnormalities such as white exsudates, furrows, edema, fixed

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rings, and stenosis as previously described[8] as either absent (0), mild (1), moderate (2), or severe (3). The total endoscopy score ranged from 0 to 21 points. During endoscopy, 2 biopsies were taken each from the distal, mid, and proximal esophagus preferably from optically visual lesions. Biopsy specimens were laid into separate tubes with neutral pH-buffered 4% paraformaldehyde and sent to the pathology department for further analysis.

Histology

Esophageal biopsies were paraffin-embedded and cut in sections for hematoxylin and eosin (HE) staining as well as IHC using standardized protocols of the pathology department. For diagnosis, on each HE staining section all levels were surveyed and the eosinophils in the most densely infiltrated area were counted in 5 consecutive hpf [Olympus BH-2 (Olympus, Tokio, Japan) magnification ×400, area of microscopic field $= 0.5\,mm^2$]. The mean and peak number of eos/hpf were calculated. Plasma cell infiltration was validated immunohistochemically using an antibody against mum1 (monoclonal mouse anti-human MUM1 protein; Agilent, Waldbronn, Germany). Ig expression of plasma cells was evaluated using antibodies against IgG (mouse anti-human, Clone IgG88; dcs diagnostics, Hamburg, Germany) and IgG4 (mouse anti-human, MRQ-44; Medac diagnostics, Wedel, Germany). The overall infiltration of plasma cells as well as the amount of IgG and IgG4-positive plasma cells were counted per hpf in the epithelium and in the subepithelial layers of EoE patients before and after topical steroid therapy as well as in GERD patients.

Serum Analysis

Venous blood was drawn prior endoscopy from all EoE patients before and after therapy and from all GERD patients at baseline. Samples for serum marker analysis were collected in tubes containing silicate-coated granules (S-Monovette Serum; Sarstedt, Germany). Serum was obtained by centrifugation at 1.000*g* for 10 minutes at 4°C. All serum samples were stored frozen at −20°C until further analysis. Before analysis samples were checked for amount and quality and insufficient samples were excluded from further analysis. Serum values for total IgG4 were measured by ELISA (Thermo Fisher, Wesel, Germany). Total IgE as well as IgE and IgG4-specific subclasses for α-lactalbumin, β-lactoglobulin, casein, wheat, egg, peanut, soy, crab, birch pollen, and phleum pratense were measured by the ImmunoCap System (Phadia, Uppsala, Sweden).

Statistical Analysis

Statistical analysis was carried out using Prism GraphPad, Version 7. For quantitative data mean and SD are presented, if not indicated differently. Comparison of data was carried out using 2-sided *t* tests. A $P < 0.05$ was considered as significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

Patient Characteristics

In total, 20 adult patients with active EoE were prospectively included in this study. Three blood samples were incomplete or insufficient, so 17 patients were included for further investigations (n = 17, 94% male, 100% white, median age 32 y with a SD of 10.8 y). Symptoms have been present for a mean of 9.7 years (SD, 7.3 y) before treatment with local corticosteroids. In addition, 14 GERD patients were included as controls (n = 14, 50% male, 100% white, median age 47 y, SD of 10.4 y).

Clinical, Endoscopic, and Histologic Response

In total, 15 of 17 patients showed complete histologic remission after 8 weeks of topical steroid therapy (51.9 eos/hpf before vs. 6.4 eos/hpf after therapy; $P < 0.0001$) (Fig. 1A). Also, the dysphagia score significantly improved (4.0 before vs. 0.65 points after therapy; $P < 0.0001$) (Fig. 1B). The total endoscopic intensity score decreased significantly from baseline to end of treatment (5.7 before vs. 2.0 points after therapy; $P < 0.001$).

Serum Levels of Total IgG4 and IgE

EoE patients had significant higher IgG4 serum levels than GERD patients at baseline (121.0 ± 68.1 vs. 71.1 ± 49.9 mg/dL; $P = 0.038$, normal reference values 10 to 140 mg/dL). IgE levels were higher in EoE than in GERD patients, but not at a significant level (534.4 ± 914.1 vs. 131.3 ± 345.7 kU/L; $P = 0.141$, normal reference values 25 to 100 kU/L).

A slight but significant decrease of total IgG4 serum levels in EoE patients could be measured after treatment (121.0 ± 68.1 vs. 104.2 ± 61.3 mg/dL; $P = 0.019$). There was no significant change of IgE serum levels (534.4 ± 914.1 vs. 508.6 ± 846.8 kU/L; $P = 0.103$) (Figs. 2A–C).

Specific Subclasses for IgE and IgG4

All measured specific IgE serum levels except for casein were slightly higher in EoE than in GERD patients but only

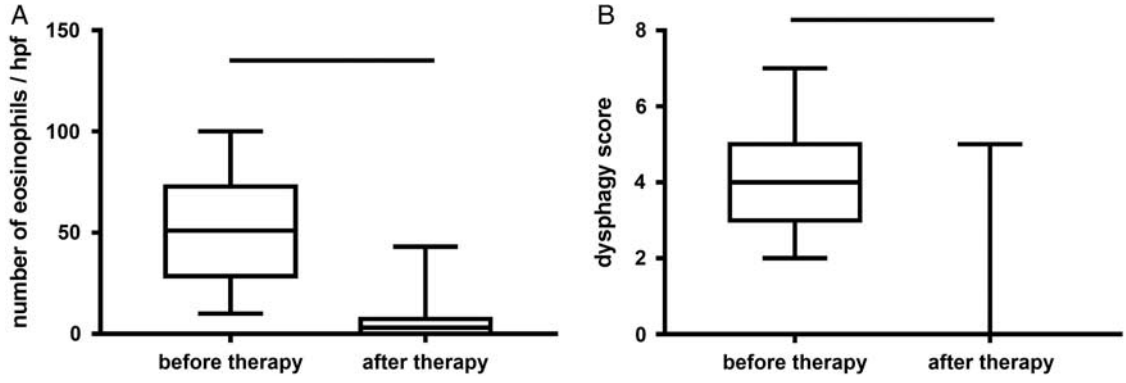

FIGURE 1. A significant reduction in eosinophil count ($P < 0.0001$) (A) and dysphagia score ($P < 0.0001$) (B) could be observed in EoE patients after topical steroid therapy.

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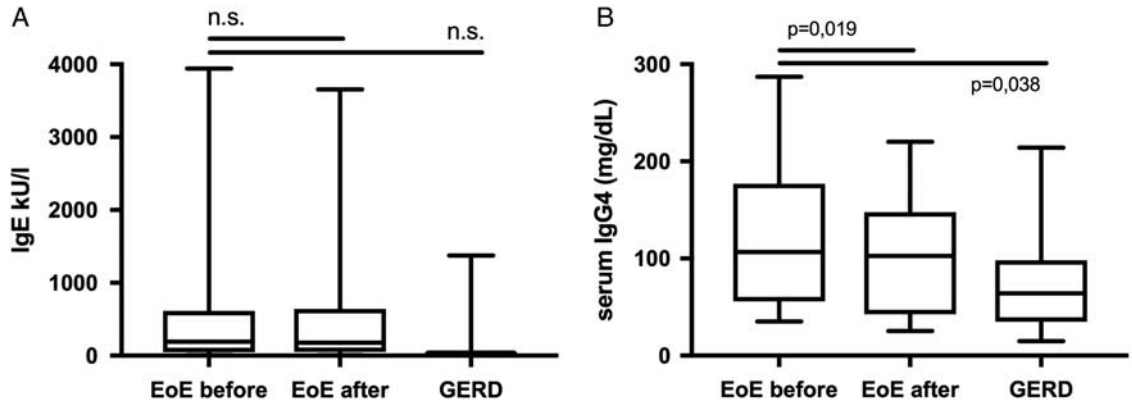

FIGURE 2. Serum IgE levels show no significant difference before and after topical steroid therapy in EoE patients (A), whereas serum IgG4 levels are significantly reduced (B). EoE indicates eosinophilic esophagitis; GERD, gastroesophageal reflux disease; NS, not significant.

statistically significant for phleum pratense. Specific IgE remained at the same level in EoE patients after budesonide therapy except for phleum pratense. In contrast, all measured specific IgG4 serum levels were significantly higher in EoE patients than in GERD patients and in EoE patients a decrease in all specific IgG4 levels except for phleum pratense were observed under therapy (Table 1 and Fig. 3).

IHC Analysis

Staining for Mum1 showed a dense infiltration of plasma cells in EoE patients at baseline (Fig. 4B). Also, a strong IgG4 expression was seen before therapy, whereas almost no signal for IgG4 could be detected after therapy (Figs. 4C, F). IgG4 could be detected in intercellular granules as well as intracellular with stronger expression of IgG4 in the subepithelial stroma than in the papillae of the lamina propria.

The count of plasma cells was significantly reduced in EoE patients after therapy (46.4 ± 25.2 before vs. 0.9 ± 1.1 after therapy; $P < 0.001$ in the stroma and 20.7 ± 25.8 before vs. 0.4 ± 0.7; $P < 0.001$ after therapy in papillae of the lamina propria), as proven through mum1 staining. Also, the count of IgG4-positive plasma cells was significantly reduced (29.1 ± 26.7 before vs. 0.1 ± 0.3; $P < 0.001$ after therapy in the stroma and 7.0 ± 15.1 before vs. 0.1 ± 0.3, not significant after therapy in the papillae of the lamina propria). In GERD patients, only singular plasma cells without expression of IgG4 were detected (Fig. 5).

TABLE 1. Serum Concentrations of Serum Immunoglobins

Serum Concentrations of Specific Subclasses for IgE and IgG4	EoE Before	EoE After	GERD	*P* (EoE Before/After)	*P* (EoE Before/GERD)
IgE total (kU/L)	534.4 ± 914.1	508.6 ± 846.8	131.3 ± 345.7	0.103	0.141
α-Lactalbumin	1.38 ± 3.65	1.57 ± 4.02	0.13 ± 0.41	0.554	0.199
β-Lactoglobulin	0.8 ± 2.8	0.9 ± 3.3	0.4 ± 1.4	0.503	0.567
Casein	0.4 ± 0.8	0.4 ± 0.8	0.9 ± 3.4	0.351	0.630
Wheat	1.4 ± 2.2	1.7 ± 2.5	0.3 ± 1.1	0.053	0.093
Egg	0.9 ± 2.4	1.1 ± 2.9	0.2 ± 0.9	0.177	0.299
Peanut	5.74 ± 17.20	1.60 ± 3.62	0.09 ± 0.25	0.279	0.253
Soy	1.03 ± 2.54	1.20 ± 2.95	0.02 ± 0.04	0.283	0.150
Crab	0.76 ± 1.94	0.93 ± 2.43	0.0.3 ± 0.09	0.453	0.159
Birch pollen	12.25 ± 21.65	6.16 ± 10.35	2.91 ± 5.53	0.875	0.269
Phleum pratense	12.24 ± 15.65	12.90 ± 15.97	0.45 ± 1.09	0.034*	0.017*
IgG4 total (mg/dL)	121.0 ± 68.1	104.2 ± 61.3	71.2 ± 49.9	0.019*	0.038*
α-lactalbumin	8.56 ± 5.94	7.64 ± 5.26	2.90 ± 4.71	0.033*	< 0.0001***
β-lactoglobulin	9.71 ± 8.02	9.34 ± 7.70	2.61 ± 5.40	0.031*	0.0003***
Casein	12.30 ± 8.31	8.25 ± 7.07	3.02 ± 5.29	0.025*	0.0002***
Wheat	8.98 ± 8.36	6.38 ± 7.26	2.60 ± 5.77	0.079	0.007**
Egg	7.17 ± 4.84	6.27 ± 5.41	2.78 ± 3.77	0.172	0.013*
Peanut	2.23 ± 4.22	1.42 ± 2.05	0.27 ± 0.48	0.113	0.085
Soy	0.97 ± 1.30	0.81 ± 1.22	0.20 ± 0.22	0.017*	0.034*
Crab	0.45 ± 1.26	0.30 ± 0.82	0 ± 0	0.157	0.180
Birch pollen	0.67 ± 0.62	0.51 ± 0.62	0.31 ± 0.60	0.031*	0.182
Phleum pratense	0.48 ± 0.40	0.50 ± 0.62	0.15 ± 0.16	0.702	0.013*

Values are expressed as mean ± SD.
After indicates after steroid therapy; Before, before steroid therapy; EoE, eosinophilic esophagitis; GERD, gastroesophageal reflux disease.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

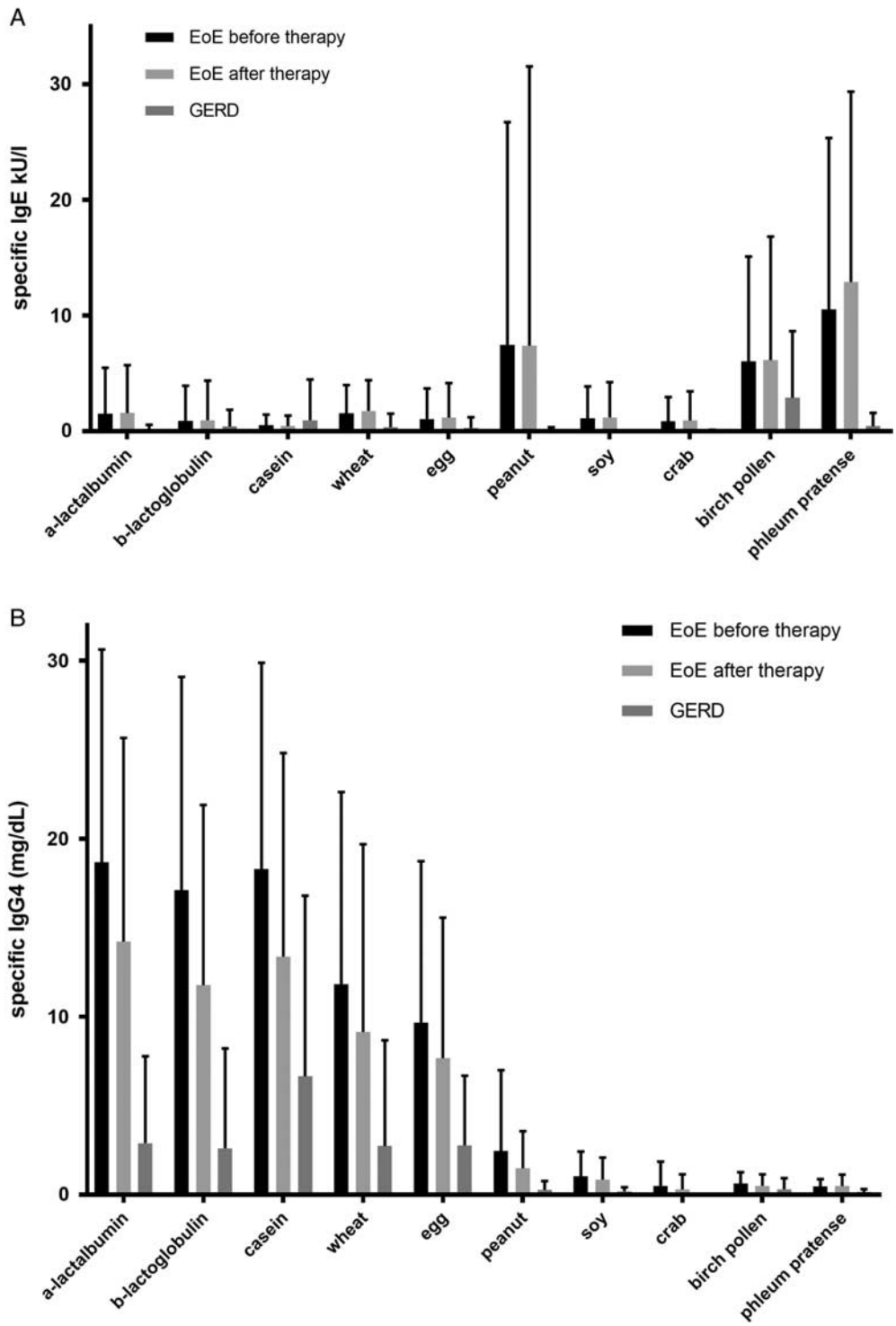

FIGURE 3. A, Specific serum IgE levels against 10 allergens in EoE and GERD patients. B, Specific serum IgG4 levels of these allergens in EoE and GERD patients. Significance levels are shown in Table 1. EoE indicates eosinophilic esophagitis; GERD, gastroesophageal reflux disease.

DISCUSSION

EoE is a chronic immune disease that can be effectively treated with topical steroids[1] which could also be shown in our study. After 8 weeks of budesonide treatment infiltration of eosinophils decreased in 15/17 patients associated with an improvement of the dysphagia score. Overall, our patient collective can be regarded as representative for an effective therapy.

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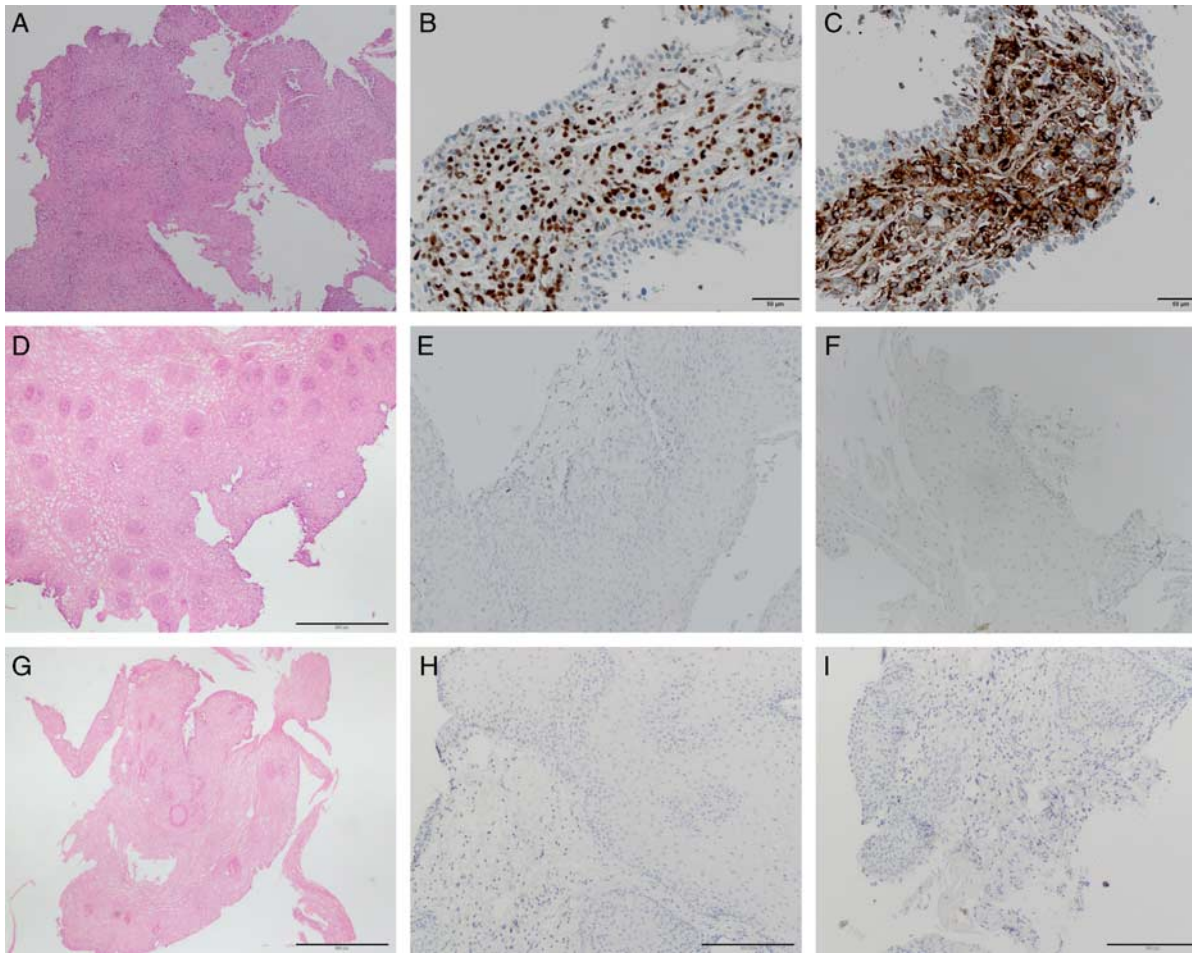

FIGURE 4. High infiltration of eosinophils in the esophageal epithelium and IgG4-positive plasma cells mainly located in the lamina propria mucosae in EoE patients before therapy (A: HE stain, ×10 magnification; B: mum1, ×20: C: IgG4, ×20). After therapy eosinophils and IgG4-positive plasma cells disappear (D: HE stain, ×10; E: mum1, ×20; F: IgG4, ×20). In GERD patients no eosinophils and only singular plasma cells without IgG4 expression are detected (G: HE stain, ×10, H: mum1, ×20; I: IgG4; ×20). EoE indicates eosinophilic esophagitis; GERD, gastroesophageal reflux disease; HE, hematoxylin and eosin.

The inflammation in EoE is predominantly eosinophilic but it is also characterized by increased number of T cells and mast cells, as well as high expression of interleukin (IL)-4, IL-5, and IL-13, showing a distinct Th2-pattern.[9–11] In experimental models it was shown that eotaxin-3 and IL-5 were crucial for the recruitment of eosinophils as well as for epithelial hyperplasia.[12] By treatment with corticosteroids expression of eotaxin-3, IL-5, and IL-13 is reduced, thus leading to a decrease of eosinophil count in the mucosa.[13]

Early studies have shown elevated levels of IgE as well as an association with atopy and sensitization to food, thus suggesting that food proteins play an important role.[9] This was underlined by the findings that food elimination and elemental formulas showed improvement in children as well in adults.[14,15] However, specific skin-prick test-directed as well as blood IgE-directed diets could induce remission only in the minority of patients and some patients with typical clinical manifestation of EoE did not show high levels of IgE.[16,17] In addition, the fact that the IgE antibody omalizumab did not show any positive effects on the disease[5,18] raised doubts on a solely IgE-driven pathogenesis of EoE. This led to a possible change of paradigm in EoE from an IgE-driven to an IgG4-driven disease.

Zukerberg et al[19] detected a strong intercellular staining of IgG4 in esophageal biopsies of adult EoE patients. These were proven to be immune complexes in electron microscopy. In addition, almost all plasma cells were IgG4-positive, with higher concentrations in deeper mucosal layers and in the lamina propria. Also, in our study we were able to detect high amounts of IgG4 plasma cells as well as intercellular IgG4 granula. We found that EoE patients had 25× higher counts of plasma cells and 145× higher counts of IgG4-positive plasma cells than GERD patients mainly located in the subepithelial stroma of the lamina propria. Clayton and colleagues compared tissue from patients fulfilling the criteria for EoE showing also high amounts of IgG4-positive plasma cells in the submucosa to healthy control tissue, which also lacked IgG4-positive plasma cells. Very recently Rosenberg et al were able to show that esophageal IgG4 levels correlate with histopathologic and transcriptomic features in pediatric EoE patients.[20]

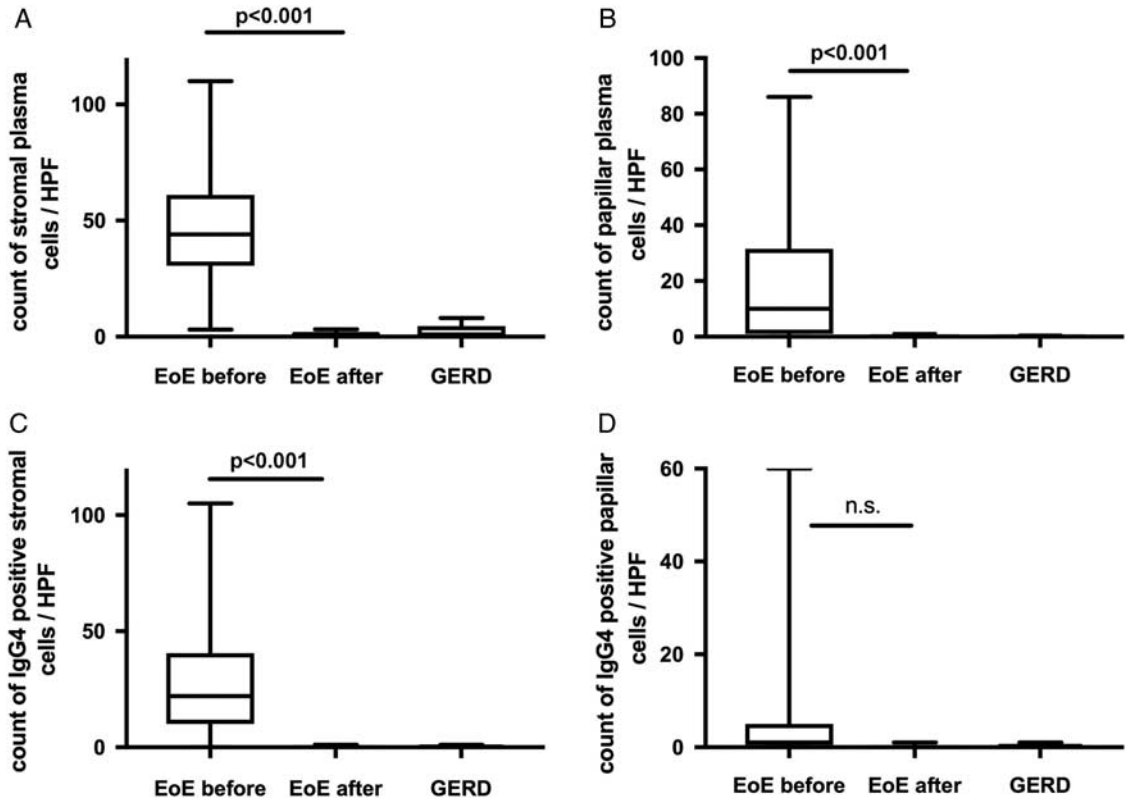

FIGURE 5. Overall and IgG4-positive plasma cells are strongly reduced in the esophagus after steroid therapy. Expression is higher in the subepithelial stroma of the lamina propria (A, C) than in the papillae (B, D). EoE indicates eosinophilic esophagitis; GERD, gastroesophageal reflux disease; HPF, high power field; NS, not significant.

Mohammad et al[21] could show that IgG4-positive plasma cells are higher in pediatric EoE patients, especially in patients with a documented food allergy.

Until now, the expression of IgG4-positive cells after topic steroid therapy has scarcely been investigated which was criticized by Philpott et al[22] who feared a too fast paradigm shift away from IgE. The current study is now one of the first looking also on the effect of therapy: Under topical steroids the count of IgG4-positive plasma cells in the tissue was almost 300× reduced and serum IgG4 levels also slightly decreased, but not serum IgE levels. Wright et al[23] reported recently that EoE subjects had elevated esophageal and plasma food-specific IgG4-values which decreased in response to dietary elimination.[23] These findings underline the hypothesis that EoE seems to be a chronic immune disease mediated more by IgG4 than by IgE.

Similar to systemic IgG4-related diseases, EoE responds to steroids and is associated with atopy, infiltration of eosinophils, numerous IgG4 plasma cells, granular IgG4 deposits, fibrosis and is male predominant.[5,24] However, compared with systemic IgG4-related disease in which IgG4 levels of >200 mg/dL are regularly seen, lower levels of systemic IgG4 could be measured in our cohort of EoE patients with more localized involvement. This effect can be possibly explained with smaller affected tissue compartment with IgG4-positive cells. Therefore, it can be postulated that EoE is rather IgG4-associated, not IgG4-related.[5]

In EoE evaluation of therapy success is usually done via biopsies from the esophagus, which demands invasive endoscopy. Until now, no valid biomarker for monitoring of therapy success has been found.[25] We have already shown that so far, the absolute blood eosinophil count seems to be the most valuable one as it shows correlation with the esophageal eosinophil density.[26] Recently it was shown that IgG4 can serve as an adjunctive marker to distinguish EoE from GERD as in 76% of the investigated cases intrasquamous extracellular IgG4 deposits were proven by IHC but in none of the GERD cases.[19] Also, in our study we were able to show that the count of IgG4-positive plasma cells was 145× higher than in GERD patients. Therefore, IgG4 staining might be valuable for distinction between EoE and GERD, especially in case of EoE with lower infiltration of eosinophils. Serum IgG4 might also be used as a noninvasive marker to distinguish EoE from GERD. Still, in EoE IgG4 does also not seem convenient for monitoring of therapy success, as mentioned before serum levels of IgG4 remain comparably low and do not necessarily exceed normal values probably due to tissue involvement.

As EoE is also related with food allergies, we measured serum levels of specific IgE and IgG4 against the 10 most prevalent food and aeroallergens. Only IgE against α-lactalbumin and phleum pratense were significantly higher in EoE than in GERD patients. Serum IgE levels did not differ from each other before and after therapy with budesonide. This could be again an indicator that EoE is not a regular food allergy but rather an IgG4-associated disease. It has been speculated that EoE might be IgE-associated or mediated initially but then becomes an IgG4-associated process with repeated trigger food exposure.[5] Although we

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have proven a statistically significant association between EoE and some specific food-related IgG4, these seem to be limited to predict trigger foods.[27]

In conclusion, our study gives further evidence that EoE is rather an IgG4-associated than an IgE-mediated disease and local IgG4 staining as well as systemic IGG4 serum levels might be helpful to distinguish EoE from GERD.

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