

# Gene expression and clinical outcomes after dietary treatment for eosinophilic esophagitis: a prospective study

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## Abstract

**Background:** Eosinophilic esophagitis (EoE) is an allergen-mediated disease and elimination diets have proven to be effective to obtain clinical and histological remission. However, the effect of elimination diets on specific EoE transcripts and their clinical correlates is relatively unknown. The main aim of the study was to evaluate the effect of dietary treatment (four-food elimination diet [FFED]) with or without addition of amino acid-based formula (AAF) on a variety of pro-/anti-inflammatory, epithelial/barrier function and remodeling/fibrosis-related markers of disease activity and clinical correlates (eosinophils, symptoms, and endoscopic signs) in adult EoE patients.

**Methods:** We conducted an analysis of biopsy samples and data collected during a randomized controlled trial with an elimination diet in adult patients with active EoE ( $\geq 15$  eosinophils [eos] per high-power field [hpf]). Demographics, symptoms (SDI-score), endoscopic signs (EREFS) and peak eosinophil counts/hpf were recorded at baseline and after 6 weeks of treatment. Transcripts of 10 indicated genes were measured (qPCR) and compared to clinical correlates at baseline and after treatment.

**Key Results:** Forty patients (pooled FFED + FFED + AAF) (60% male, age 34.5 (interquartile range [IQR] 29–42.8 years) completed the diet. Peak eosinophil counts/hpf, symptoms and endoscopic signs were significantly decreased after 6 weeks dietary treatment. DSG-1 levels were significantly upregulated from baseline to week 6, whereas IL-13, CAPN-14, IL-5, IL-10, CCL-26, POSTN, TSLP, CPA-3, and TGF- $\beta$  were significantly downregulated after 6 weeks of diet (all;  $< 0.01$ ). Prior to treatment, upregulation of CAPN-14 and lower levels of DSG-1 were associated with clinical fibrotic phenotypes, whereas upregulation of IL-10 was linked to food impaction phenotypes.

**Conclusion:** These findings strongly suggest that elimination diets, besides a clinical and histological response, are associated with a broad transcriptional response at the level of the esophageal epithelium.

**Abbreviations:** AAF, amino acid-based formula; CAPN-14, Calpain; CI, confidence intervals; CPA-3, carboxypeptidase 3A; DSG-1, Desmoglein; EoE, eosinophilic esophagitis; eos, eosinophils; EREFS, Endoscopic Reference Score; FFED, four-food elimination diet; GERD, gastroesophageal reflux disease; hpf, high power field; OR, odds ratio; POSTN, periostin; PPI, proton pump inhibitor; qPCR, quantitative real-time PCR; SDI, Straumann Dysphagia Instrument; TGF- $\beta$ , transforming growth factor- $\beta$ ; Th2, T-helper type 2; TSLP, Thymic stromal lymphopoietin.

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## KEYWORDS

eosinophilic esophagitis, esophageal eosinophilia and allergy

## 1 | INTRODUCTION

Eosinophilic esophagitis (EoE) is an allergen/immune-mediated esophageal disorder, characterized by symptoms of esophageal dysfunction (i.e., dysphagia and food impaction) and eosinophilic infiltration of the esophageal epithelium.<sup>1,2</sup> There has been a growing understanding of EoE pathogenesis following its first description in the early 1990s.<sup>3,4</sup> Overall, the evolution of EoE is a multifactorial interplay of genetics, environmental, and host immune system factors that are involved in multiple pathways.<sup>5,6</sup> The proposed immunological mechanism is illustrated by an immune response that is primarily regulated by T-helper type 2 cells (Th2) against food- (and aero) allergens. Thymic stromal lymphopoietin (TSLP) is released by activated esophageal epithelial cells after allergen exposure and has an important role in promoting Th2 differentiation.<sup>5</sup> Activated dendritic cells initiate T-cell polarization to Th2 cells, that serve as a source of pro-inflammatory cytokines, such as interleukin (IL)-5 and IL-13 or products induced by these cytokines (IL-13-induced eotaxin-3 [CCL26]).<sup>7</sup> Genes specific to mast cells, such as those that encode carboxypeptidase 3A (CPA-3), were also found to be highly expressed in the EoE transcriptome.<sup>8</sup> Locally activated eosinophils and mast-cells produce Transforming Growth Factor (TGF)- $\beta$ , a key cytokine for epithelial cell transformation and fibrosis.<sup>9</sup> Moreover, IL-13 induced calpain (CAPN)-14—which is specifically found to be overexpressed in EoE patients—downregulates desmoglein (DSG)-1, a barrier protein, by that disrupting the esophageal epithelial barrier.<sup>10</sup> Loss of DSG-1 may also potentiate allergic inflammation through the induction of pro-inflammatory mediators, such as periostin (POSTN).<sup>11,12</sup> Finally, the potent anti-inflammatory cytokine IL-10 seems to be of interest since it was found to be upregulated in pediatric EoE patients compared with controls, by that linking this pleiotropic immunoregulatory cytokine to EoE pathogenesis.<sup>13</sup> Diets have proven to be effective in EoE and target the adaptive immune system (i.e., suppression of antigen-driven T-cell response by elimination of culprit foods) with no modification of signaling pathways or inflammatory cell apoptosis as often occurs after steroids or biological targets.<sup>14–17</sup> There is a relative scarcity of data evaluating the effect of dietary treatment on gene expression patterns in adult EoE, in particular, in the context of clinical features.<sup>5,7,18</sup> Considering its heterogeneous disease presentation and the clinical impact of fibrotic complications, personalized treatment strategies based on EoE endotypes being more or less fibrotic may be needed. Therefore, we aimed to investigate the effect of a four-food elimination diet (FFED) (i.e., exclusion of gluten, milk, soy, and eggs) on multiple pro-/anti-inflammatory (IL-5, IL-13, TSLP, POSTN CPA-3, CCL-26, and IL-10), epithelial/barrier function (DSG-1, CAPN-14), and remodeling/fibrosis (TGF- $\beta$ )-related markers of disease activity and clinical correlates (eosinophils, symptoms, and endoscopic signs) in adult EoE patients.

### Key points

- There is a relative scarcity of data evaluating the effect of dietary treatment on gene expression patterns in adult EoE, in particular, in the context of clinical features.
- Multiple pathways that are leading to this common disease state are affected after dietary treatment, with significant changes of gene expression markers related to inflammation (IL-5, IL-13, TSLP, POSTN CPA-3, CCL-26, and IL-10), epithelial/barrier function (DSG-1 and CAPN-14) and remodeling/fibrosis (TGF- $\beta$ )
- Upregulation of CAPN-14 and lower levels of DSG-1 were associated with “fibrotic” phenotypes, whereas upregulation of IL-10 was linked to “food impaction” phenotypes.
- These findings strongly suggest that elimination diets, besides a clinical and histological response, are associated with a broad transcriptional response at the level of the esophageal epithelium and provide a foundation for the future mechanistic studies.

## 2 | METHODS

### 2.1 | Study design and patients

We conducted an analysis of biopsy samples and data collected during a randomized controlled trial of adult EoE patients. The parent study, of which details have been described previously,<sup>19</sup> included patients from the outpatient clinic of the Amsterdam UMC motility center between December 2017 and January 2020.<sup>19</sup> Adult patients ( $\geq 18$  years) were eligible for study inclusion if EoE was diagnosed per consensus guidelines (i.e., presence of symptoms related to esophageal dysfunction and  $\geq 15$  eosinophils [eos] per microscopic high-power field [hpf] at baseline biopsy).<sup>20</sup> Patients were excluded if they had severe comorbidity scored as American Society of Anesthesiologists (ASA) Physical Classification System class IV or higher, the inability to stop anti-inflammatory drugs (i.e., topical or systemic steroids, leukotriene inhibitors, or monoclonal antibodies), a recent history of gastrointestinal cancer or major Gastrointestinal surgery. This study was approved by the Medical Ethics Committee of the Amsterdam UMC and prospectively registered in the Dutch trial registry NL6014 (NTR6778). Written informed consent was obtained from all participants before taking part and a unique study ID was given to ensure anonymity.

## 2.1.1 | Study protocol

After informed consent was obtained, participants underwent an upper endoscopy with biopsy sampling at baseline and after 6 weeks of dietary treatment. Histological features, endoscopic signs, and symptoms were evaluated at baseline and at week 6. If consent was obtained and eligibility was confirmed after baseline upper endoscopy, patients were randomized (1:1 fashion) to either a four-food elimination diet (FFED) (i.e., exclusion of gluten, milk, soy, and eggs) or a FFED with the addition of an amino acid-based formula (AAF) providing 30% of patients' daily energy needs (FFED + AAF) by using a blocked randomization protocol (i.e., sealed envelopes). Comparison of FFED + AAF vs. FFED in the parent study did not show a significant difference between both groups on clinical, endoscopic, and histological outcomes. To evaluate the general effect of an elimination diet on gene expression in a large sample of EoE patients, data of both groups were pooled in this follow-up study. In our trial, trends toward lower histological disease activity in patients treated with the FFED + AAF compared with those treated with FFED alone were observed.<sup>19</sup> Therefore, a subgroup analysis was performed on the treatment effect of the AAF added to the FFED on gene expression levels.

Biopsies that were sampled prior and after 6 weeks of dietary treatment were used to measure gene expression related to disease activity (i.e., eosinophils, symptoms, and endoscopic signs).

## 2.2 | Study procedures

### 2.2.1 | Clinical data, sample collection, and clinical subgroup definition

Demographics, symptoms, and endoscopic data were recorded prospectively by using standardized case report forms. Symptoms of dysphagia were evaluated by means of the Straumann Dysphagia Instrument (SDI) measure.<sup>21</sup> This measure ranges from 0 to 9 and consists of 2-items (dysphagia frequency [0–4] and dysphagia intensity [0–5]). A “clinical response” was defined as a reduction of  $\geq 3$  points of the after treatment SDI score compared with baseline.

Upper endoscopy was performed and endoscopic features of EoE were classified according to the modified Endoscopic Reference Score (EREFS) grading system.<sup>22</sup> Endoscopic features were subclassified (EREFS) as inflammatory (white exudates, edema, and linear furrows) and fibrotic (rings and strictures) signs.

During upper endoscopy, six biopsies were taken from the distal, mid, and proximal esophagus per standardized protocol. A  $\times 400$  magnification was used in order to determine the peak eosinophil count (PEC) per hpf (an area of  $0.24 \text{ mm}^2$ ). “Histological remission” after induction treatment was defined as patients achieving a PEC of  $< 15$  eos/hpf at histological assessment after diet treatment.

Clinical findings were further defined by means of clinical phenotype definition, which has been previously described by Dellon et al.<sup>23</sup> Patients presenting with symptoms of food impaction (i.e., SDI measure, item 2; dysphagia intensity of  $\geq 3$ ) were defined (yes or no) as “food-impaction” phenotypes (vs. “non-food impaction” phenotypes). Patients were defined (yes or no) as having a “fibrotic” phenotype, if endoscopically “rings” and/or “strictures” were present (i.e., EREFS fibrotic subscore  $\geq 1$ ) (vs. “non-fibrotic” phenotype). Gene expression levels were compared at 2 time points (i.e., baseline and after 6 weeks) between patients with these pre-defined clinical subgroups.

### 2.2.2 | Gene expression determination

In addition to the biopsies for histology, three more biopsies were taken from the mid esophagus during upper endoscopy at baseline and after treatment. Gene expression was measured in these esophageal samples to define overall expression levels of the indicated genes (IL-5, IL-13, TSLP, POSTN, CPA-3, CCL-26, IL-10, DSG-1, CAPN-14, and TGF- $\beta$ ). These three additional biopsies were immediately immersed in RNA stabilization reagent (RNA-later, Invitrogen/Thermo Fisher Scientific, Baltics UAB). First, the biopsies were stored for 24 h at  $4^\circ\text{C}$ , with subsequent storage at  $-80^\circ\text{C}$ . The mid-esophageal biopsies in RNA-later ( $-80^\circ\text{C}$  storage) were sent on dry ice for processing and gene expression testing to Utrecht University. Biopsies in RLT lysis buffer (Qiagen mRNeasy kit) containing 10%  $\beta$ -mercaptoethanol were homogenized by using the Precellys homogenisator (Bertin). RNA extraction was performed on homogenized specimens using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was measured by using NanoDrop One spectrophotometry (Isogen Life Sciences) and subsequently 500 ng RNA was used for cDNA synthesis by using the iScript cDNA synthesis kit (Biorad). Quantitative real-time (RT) PCR was performed on a CFX96 Touch quantitative real-time (q) PCR device (Biorad) to determine the gene expression levels measured as threshold cycles (Ct). Commercially available primers for IL-5, IL-10, IL-13, CPA-3, CAPN-14, DSG-1, CCL-26, POSTN, and TSLP were obtained (all from Biorad). RPL13A was used as a reference gene for normalization of all genes of interest (Biolegio, 5'CATAGGAAGCTGGGAGCAAG3' and 5'GCCCTCCAATCAGTCTTCTG 3') and was used to calculate normalized mRNA expression. The mRNA level was calculated with CFX manager software and corrected for the expression of RPL13A with  $100 \times 2^{\Delta(\text{RPL13A-gene of interest})}$ . Relative values of the gene of interest were calculated by extracting after treatment values by the genes of interest prior to treatment.

## 2.3 | Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics (version 25.0) (SPSS). Descriptive statistics were used to summarize all characteristics of the study sample. Categorical variables are

described as percentages and continuous variables are expressed as mean with standard deviation (SD) or median with interquartile ranges (IQR). Baseline and after treatment values within the total sample ( $n = 40$ ) or subgroups (FFED,  $n = 20$  and FFED + AAF,  $n = 20$ ) were compared by using the Wilcoxon signed-rank test for ordinal data and McNemar's test for categorical data. Normally and non-normally distributed continuous data between (clinical) (sub) groups were compared by using a  $t$ -test or Mann-Whitney  $U$ -test, if appropriate. A  $p$ -value of  $<0.05$  was considered statistically significant.

### 3 | RESULTS

#### 3.1 | Patients characteristics

Fifty-two patients were eligible for inclusion. After baseline endoscopy, 11 patients were excluded due to non-active disease ( $<15$  eos/hpf) at histological evaluation. Forty out of the 41 patients who started the diet treatment (FFED group [ $n = 20$ ] and FFED + AAF group [ $n = 21$ ]) completed the trial according to the protocol guidelines. A male predominance (60%) was confirmed with a median age of 34.5 (IQR 29–42.8) years. The majority of patients (63%) had  $\geq 2$  additional atopic comorbidities. Details on baseline characteristics of all included EoE patients who completed the 6 weeks dietary treatment are listed in [Table 1](#).

#### 3.2 | Dietary treatment effect on histological, endoscopic, and symptomatic outcomes and gene expression

##### 3.2.1 | Treatment effect on esophageal eosinophilia, symptoms, and endoscopic signs

Six weeks of dietary treatment (data pooled of FFED and FFED + AAF) reduced the median peak eosinophil count (PEC) significantly from 55.5 (IQR 41.3–93.5) to 24.5 (IQR 5–43.8) after 6 weeks ( $p < 0.001$ ) ([Table 2](#)). Fifteen patients out of the 40 (38%) had esophageal peak eosinophil counts of  $<15$  eos/hpf (i.e., histological remission) after 6 weeks of dietary treatment. Symptom severity, measured by means of the SDI score, significantly decreased from 5 (IQR 4–6) to 2 (IQR 0–4) at week 6 ( $p < 0.001$ ) ([Table 2](#)). A clinical response (i.e., reduction of  $\geq 3$  points of the SDI-score compared with baseline) was observed in 20 patients (50%) after 6 weeks of dietary treatment ([Table 2](#)). Additionally, the total EREFS score significantly decreased from 4 (IQR 3–5) to 3 (IQR 1.25–4) after 6 weeks of dietary treatment ( $p < 0.001$ ). Also significant reductions in both the inflammatory and fibrotic subscores were observed from baseline to week 6: (2 [IQR 2–3]–2 [IQR 1–2];  $p = 0.003$ ) and (2 [IQR 1–3]–1 [IQR 1–2];  $p < 0.001$ ), respectively ([Table 2](#)). More details on symptoms, endoscopic, and histological features before and after treatment are presented in [Table 2](#).

**TABLE 1** Baseline characteristics of all patients who completed the diet intervention ( $n = 40$ )

Characteristics	
Male gender, $n$ (%)	24 (60)
Age, years, median (IQR)	34.5 (29–42.8)
Race, Caucasian, $n$ (%)	38 (95)
History of allergic disease, $n$ (%)	34 (85)
Allergic rhinitis	27 (68)
Asthma	12 (30)
Atopic dermatitis	12 (30)
Food allergy	11 (28)
“Food impaction” phenotype, yes, $n$ (%)	23 (58)
Fibrotic phenotype, $n$ (%)	32 (80)
Esophageal stricture dilation, $n$ (%)	3 (8)
Previous endoscopic intervention with food bolus extraction, $n$ (%)	17 (43)
Diagnostic delay <sup>a</sup> , median (IQR)	4 (1–9)

Abbreviations: “Fibrotic” phenotype, presence of “rings” and/or “strictures” at upper endoscopy; “Food impaction” phenotype, patients presenting with symptoms of food impaction; “Inflammatory-only phenotype”, patients presenting with exudates, edema and/or furrows with no endoscopic signs of fibrotic features (i.e., rings and strictures); EoE, eosinophilic esophagitis.

<sup>a</sup>Time interval between first reported EoE symptoms and year of diagnosis.

##### 3.2.2 | Gene expression baseline/after treatment

Evaluation of gene expression in esophageal biopsy specimens at baseline and after treatment ( $n = 40$ , both groups pooled) showed significantly upregulated levels of DSG1 ( $p = 0.001$ ) ([Figure 1A](#)). This increase in DSG-1 coexisted with a decrease in IL-13 and CAPN-14 (all;  $p < 0.001$ ), which are both also involved in epithelial barrier function. In addition, the genes encoding for IL-5, IL-10, CCL-26, POSTN, TSLP, CPA-3, and TGF- $\beta$  were significantly downregulated after treatment compared with baseline (all;  $<0.01$ ) ([Figure 1B–J](#)).

##### 3.2.3 | Esophageal eosinophilia, endoscopic signs, and gene expression baseline/after treatment

Spearman's correlation analysis demonstrated a mild positive correlation for the PEC levels after treatment and mRNA expression levels of IL-5 ( $r = 0.32$ ;  $p = 0.061$ ) and a strong positive correlation for levels of CCL-26 ( $r = 0.41$ ;  $p = 0.008$ ), IL-13 ( $r = 0.5$ ;  $p = 0.002$ ), and CPA-3 ( $r = 0.4$ ;  $p = 0.01$ ) at week 6. Moreover, a significant negative correlation between peak eosinophil counts and mRNA expression levels of DSG-1 ( $r = -0.39$ ;  $p = 0.014$ ) at week 6 was observed. The expression levels of CAPN-14, IL-10, TSLP, TGF- $\beta$ , and POSTN at week 6 did not correlate with the PEC after the diet (all;  $p > 0.05$ ). In addition, a significant positive correlation was observed between the absolute change in PEC from baseline to week 6 and the relative gene

**TABLE 2** Clinical, histological, and endoscopic signs before and after treatment

	Baseline (n = 40)	Post-treatment (n = 40)	p Value
<b>Histology</b>			
Peak eosinophil counts, median (IQR)	55.5 (41.3–93.5)	24.5 (5–43.8)	<0.001 <sup>a,*</sup>
Histological remission <sup>i</sup> , yes, n (%)		15 (38)	
<b>Endoscopic signs</b>			
EREFS score (total), median (IQR)	4 (3–5)	3 (1.25–4)	<0.001 <sup>a,*</sup>
EREFS Inflammatory score, median (IQR)	2 (2–3)	2 (1–2)	0.003 <sup>a,*</sup>
EREFS Fibrotic score, median (IQR)	2 (1–3)	1 (1–2)	<0.001 <sup>a,*</sup>
<b>Symptoms</b>			
SDI-score, median (IQR)	5 (4–6)	2 (0–4)	<0.001 <sup>a,*</sup>
Clinical response <sup>ii</sup> , yes, n (%)		20 (50)	

Note: <sup>i</sup> Histological remission = patients with a peak eosinophil count of <15 eosinophils (eos) per high power field (hpf) after intervention; <sup>ii</sup> Clinical response = reduction in  $\geq 3$  points of the post-treatment SDI-score compared with baseline. <sup>a</sup> p value baseline vs. post-treatment (Wilcoxon signed-rank test); <sup>b</sup> p value baseline vs. post-treatment (McNemar test); \* p-value (two-sided) of <0.05, indicating a significant outcome.

Abbreviations: EoE, eosinophilic esophagitis; EREFS, endoscopic features are scored according to the EREFS classification and sub-classified as (i) inflammatory signs including white exudates, edema and linear furrows (ii) fibrotic signs including rings and strictures; IQR, Interquartile range; SDI, Straumann Dysphagia Instrument.

expression of CPA-3 after the diet ( $r = 0.337$ ;  $p = 0.038$ ). However, no correlations were found between the absolute change in PEC (baseline/after treatment) and the relative gene expression for the other 9 genes of the EoE-panel (all;  $p > 0.05$ ).

A significant positive correlation was observed between pre-treatment inflammatory subscores and expression levels of CPA3 ( $r = 0.33$ ;  $p = 0.045$ ), IL13 ( $r = 0.45$ ;  $p = 0.005$ ), IL5 ( $r = 0.41$ ;  $p = 0.014$ ), periostin ( $r = 0.4$ ;  $p = 0.015$ ), and CCL26 ( $r = 0.4$ ;  $p = 0.014$ ) at baseline. In addition, post-treatment levels of the inflammatory subscores also significantly correlated with mRNA expression levels of CPA3 ( $r = 0.44$ ;  $p = 0.004$ ) and CCL26 ( $r = 0.37$ ;  $p = 0.019$ ) at week 6. However, no correlations were found on the pre-/post-treatment fibrotic subscores and mRNA expression levels of all 10 genes of the EoE-panel at baseline and at week 6 (all;  $p > 0.05$ ). Additionally, no correlations were observed between the relative change of both the inflammatory and fibrotic subscores and the relative mRNA expression levels of all 10 genes of the EoE-panel from baseline to 6 weeks (all;  $p > 0.05$ ).

### 3.2.4 | Clinical phenotypes and mRNA expression

Significantly higher baseline mRNA expression levels of IL-10 were shown in 23 patients (58%) who were identified as “food impaction” phenotypes (vs. “non-food impaction” phenotypes;  $p = 0.01$ ) (Table 2, Figure 2A) indicating a role for IL-10 in this phenotype. Additionally, significantly higher baseline transcript levels of CAPN-14 and lower levels of DSG-1 were observed in 32 patients (80%) with a “fibrotic” phenotype (vs. “non-fibrotic” phenotype;  $p = 0.002$  and  $p = 0.0018$ ), respectively (Table 2, Figure 2B,C). In addition, no differences in gene expression levels of all 10 genes of

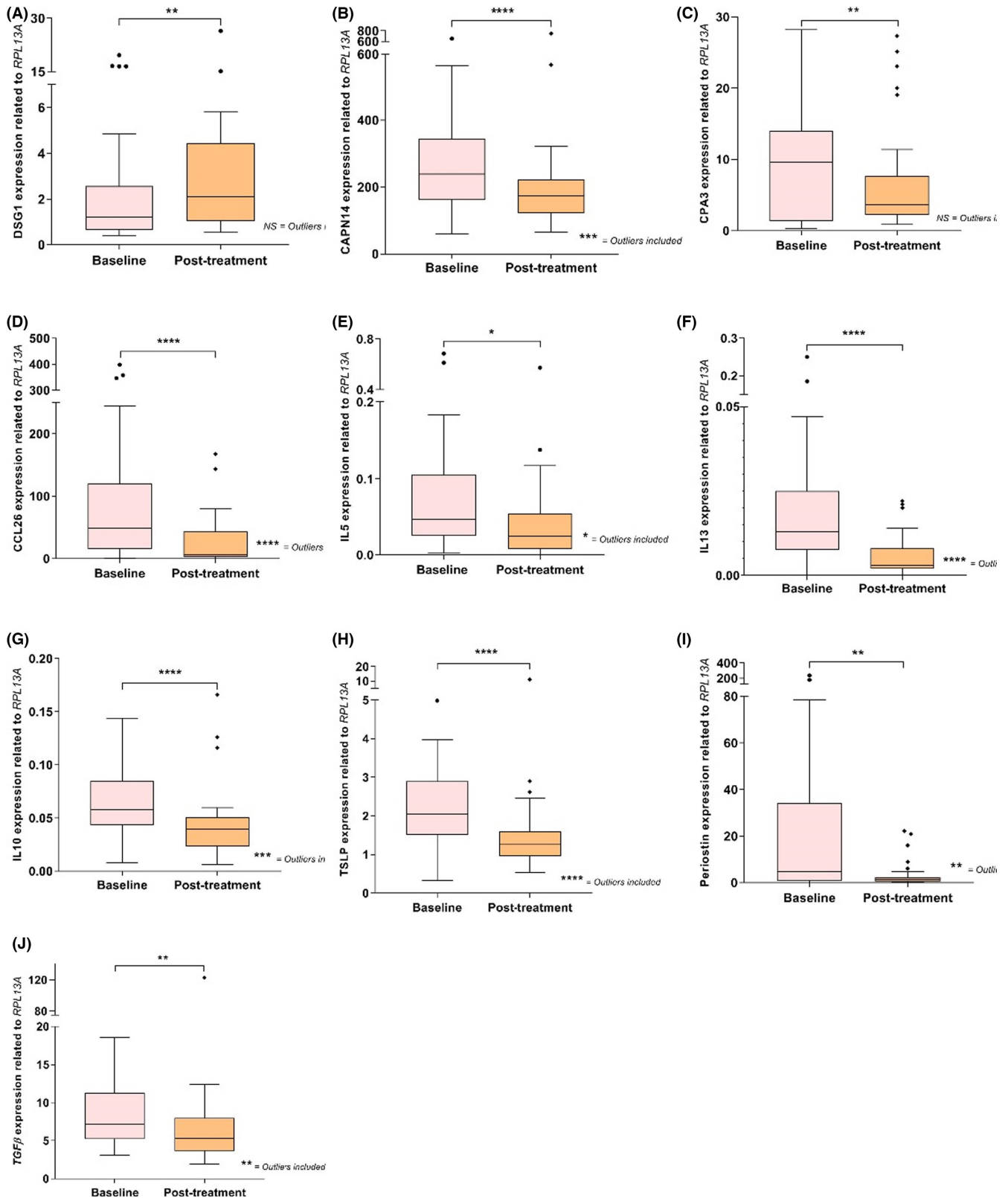
the EoE panel associated with clinical phenotypes were observed after treatment.

### 3.2.5 | Clinical and histological response and gene expression after treatment

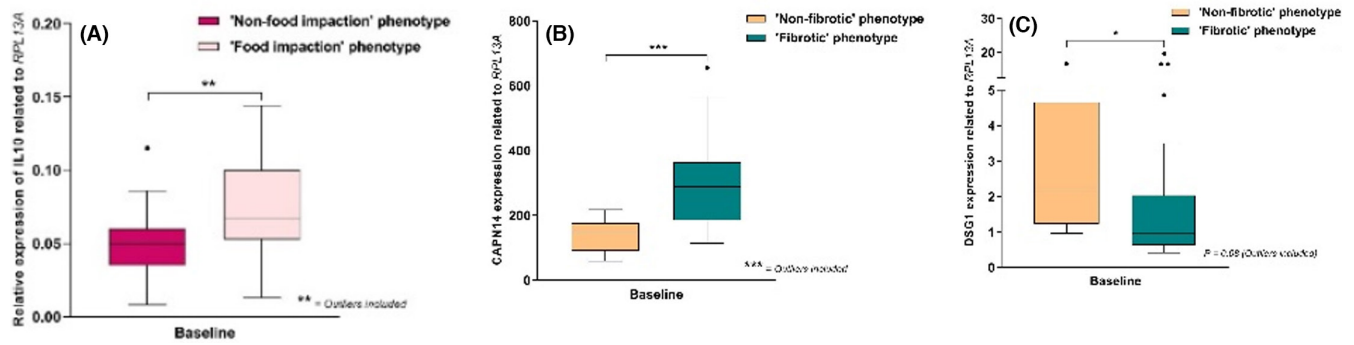
The relative mRNA expression of genes encoding for IL-13 after treatment was significantly lower in 20 patients (50%) presenting with a clinical response after the diet (vs. no clinical response;  $p = 0.006$ ) (Figure 3C). Moreover, the relative mRNA expression levels of genes encoding for IL-13 ( $p = 0.02$ ) and IL-5 ( $p = 0.02$ ) were significantly lower in the 15 patients (38%) achieving histological remission after the diet compared with those remaining with active disease (Table 2, Figure 3A,B).

### 3.3 | Subgroup analysis: Treatment Effect of AAF added to a FFED on gene expression

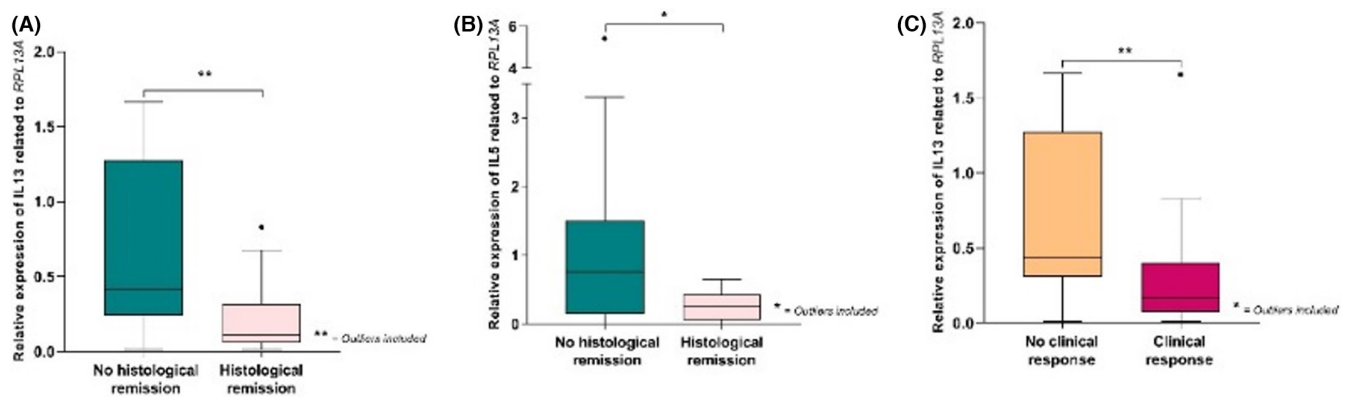
Subsequently, the patients being treated for 6 weeks with FFED ( $n = 20$ ) were compared with those treated with FFED + AAF ( $n = 20$ ) for gene expression in esophageal biopsy specimens. At baseline, inter-group comparison between patients treated with FFED or FFED + AAF showed no significant differences for transcripts of all 10 genes of our EoE-panel (all;  $p > 0.05$ ) (Figures 4A–J). The relative change in gene expression of DSG-1 in FFED + AAF-treated patients from baseline to after treatment was significantly higher compared with the relative change in FFED-treated patients after treatment ( $p = 0.04$ ) (Figure 4A). Also the relative gene expression of CPA-3 in FFED + AAF-treated patients was significantly more downregulated



**FIGURE 1** Effect of an elimination diet on the expression of genes encoding for (A) desmoglein (DSG) 1, (B) calpain (CAPN) 14, (C) carboxypeptidase (CP) A3, (D) chemokine-ligand (CCL) 26, (E) interleukin (IL) 5, (F) interleukin (IL) 13, (G) interleukin (IL) 10, (H) thymic stromal lymphopoietin (TSLP), (I) periostin, and (J) transforming growth factor (TGF)  $\beta$  pre- and post-treatment in the entire EoE sample ( $n = 40$ , both group pooled). The statistical difference between gene expression levels from baseline vs. post-treatment was calculated by means of Wilcoxon signed-rank test. EoE, eosinophilic esophagitis; NS, non-significant outcome. \*  $p$ -Value (two-sided) of  $<0.05$ , indicating a significant outcome. \*\*  $p$ -value (two-sided) of  $<0.01$ . \*\*\*  $p$ -value (two-sided) of  $<0.001$ . \*\*\*\*  $p$ -value (two-sided) of  $<0.0001$



**FIGURE 2** Expression levels of genes of interest in EoE patients ( $n = 40$ ) with different clinical phenotypes before diet intervention (A) Interleukin (IL) 10 levels in “food impaction” phenotypes vs. “non-food impaction” phenotypes (B) Calpain (CAPN) 14 levels in “fibrotic” phenotypes vs. “non-fibrotic” phenotypes (C) Desmoglein (DSG) 1 levels in “fibrotic” phenotypes vs. “non-fibrotic” phenotypes. EoE, eosinophilic esophagitis, “Food impaction” phenotype, patients presenting with symptoms of food impaction, “Fibrotic” phenotype, presence of “rings” and/or “strictures” at upper endoscopy. The statistical difference of gene expression levels at baseline between clinical subgroups was calculated using a  $t$ -test or Mann–Whitney  $U$  test, as appropriate. \*  $p$ -Value (two-sided) of  $<0.05$ , indicating a significant outcome. \*\*  $p$ -value (two-sided) of  $<0.01$ . \*\*\*  $p$ -value (two-sided) of  $<0.001$ . \*\*\*\*  $p$ -value (two-sided) of  $<0.0001$



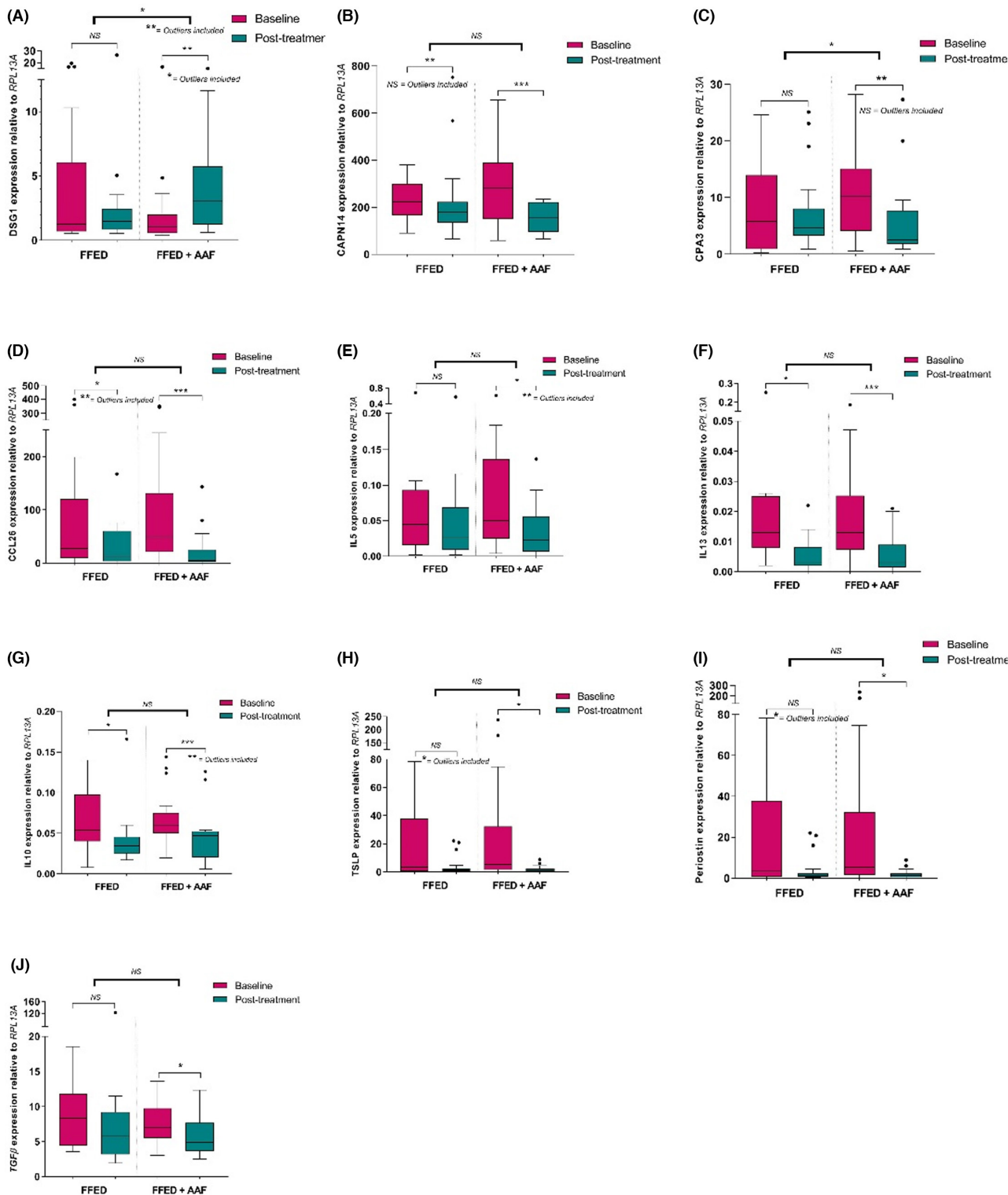
**FIGURE 3** Relative expression of genes of interest in EoE patients ( $n = 40$ ) achieving histological remission vs. no histological remission; (A) interleukin (IL) 13, (B) interleukin 5 and in EoE patients ( $n = 40$ ) showing a clinical response vs. no clinical response; (C) interleukin 13 after diet intervention. EoE, eosinophilic esophagitis, Histological remission =  $<15$  eosinophils (eos) per high power field (hpf) after intervention at histological assessment. Clinical response = reduction of  $\geq 3$  points of the Straumann Dysphagia Instrument (SDI) score at week 6 compared to baseline. The statistical difference of gene expression levels after treatment between clinical subgroups was calculated by using a  $t$ -test or Mann–Whitney  $U$  test, as appropriate. \*  $p$ -value (two-sided) of  $<0.05$ , indicating a significant outcome. \*\*  $p$ -value (two-sided) of  $<0.01$ . \*\*\*  $p$ -value (two-sided) of  $<0.001$ . \*\*\*\*  $p$ -value (two-sided) of  $<0.0001$

compared with FFED-treated patients after treatment ( $p = 0.003$ ) (Figure 4C). The relative change in expression levels from baseline to week 6 for the other 8 genes of the EoE panel was similar between both groups (all;  $p > 0.05$ ) (Figure 4B, D–J).

Within group comparison showed a significant upregulation of mRNA expression levels of DSG-1 from baseline to week 6 in patients treated with FFED + AAF ( $p = 0.001$ ) (Figure 4A). In addition, a significant reduction in transcripts for CAPN-14, DSG-1, CPA-3, CCL-26, IL-5, IL-13, IL-10, TSLP, POSTN, and TGF- $\beta$  was observed after treatment with FFED + AAF (all;  $p < 0.05$ ) (Figure 4B–J). Moreover, comparison from baseline to after treatment in patients treated with FFED alone showed significantly decreased mRNA expression levels of CAPN-14, CCL-26, IL-13, and IL-10 after 6 weeks (all;  $p < 0.05$ ) (Figure 4B, D, F, G), whereas no differences in transcripts of DSG-1, CPA-3, IL-5, TSLP, POSTN, and TGF- $\beta$  were observed after treatment (all;  $p > 0.05$ ) (Figure 4A–C, E, G, H–J).

## 4 | DISCUSSION

This is the first prospective study evaluating the effect of (2 types of) dietary treatment on the changes in 10 indicated gene expression markers related to disease activity and clinical outcomes (eosinophils, symptoms, and endoscopic signs) in adult EoE patients. Our study shows a broad transcriptional response on the esophageal epithelium, targeting multiple key pathways that are leading to this common disease state. We observed that transcript levels of proteins associated with epithelial/barrier function, such as DSG-1, were significantly upregulated after 6 weeks of dietary treatment. Moreover, transcripts of multiple pro-inflammatory (IL-5, IL-13, TSLP, POSTN CPA-3, and CCL-26), the pleiotropic cytokine IL-10 as well as markers related to epithelial/barrier function (CAPN-14) and remodeling/fibrosis (TGF- $\beta$ ) were significantly downregulated after treatment.



**FIGURE 4** Intra-group comparison of the expression of genes encoding for (A) desmoglein (DSG) 1, (B) calpain (CAPN) 14, (C) carboxypeptidase (CP) A3, (D) chemokine-ligand (CCL) 26, (E) interleukin (IL) 5, (F) interleukin (IL) 13, (G) interleukin (IL) 10, (H) thymic stromal lymphopoietin (TSLP), (I) periostin, and (J) transforming growth factor (TGF)  $\beta$  pre- and post-treatment. In addition, inter-group comparison of the relative mRNA expression levels of the 10 genes of the EoE-panel from baseline to 6 weeks is presented. EoE, eosinophilic esophagitis; FFED + AAF, four-food elimination diet with addition of amino acid-based formula; FFED, four-food elimination diet; NS, non-significant outcome. The statistical difference between gene expression levels from baseline vs. post-treatment within subgroups was calculated by means of Wilcoxon signed-rank test. Inter-group comparison of the relative mRNA expression levels of the 10 genes of the EoE-panel from baseline to 6 weeks between FFED and FFED + AAF was calculated by using a t-test or Mann-Whitney *U* test, as appropriate. \* *p*-value (two-sided) of <0.05, indicating a significant outcome. \*\* *p*-value (two-sided) of <0.01. \*\*\* *p*-value (two-sided) of <0.001. \*\*\*\* *p*-value (two-sided) of <0.0001



Given the paucity of data in the literature on the effect of dietary treatment on gene expression profiles in EoE, our findings are not directly comparable to previous studies. Warners et al. reported a similar significant reduction in mRNA expression levels of Th2 cytokines (IL-5 and IL-13) and pro-inflammatory mediators such as TSLP and POSTN in adult EoE patients after 4 weeks of an exclusively elemental diet.<sup>24,25</sup>

Additionally, in our study, significant lower transcript levels of Th2 cytokines (IL-5 and IL-13) were seen in patients achieving histological remission (i.e., <15 eos/hpf) compared with those with no histological remission after 6 weeks of diet. Moreover, gene expression levels of IL-5, IL-13, CCL-26, and CPA-3 after the diet showed positive correlations with peak eosinophil counts. These effects of the dietary treatment are in line with previously reported elements of EoE pathogenesis.<sup>6-8,10-12,26</sup> The major effector cytokine IL-13 stimulates epithelial production of eotaxin-3 (CCL-26), a potent chemoattractant for eosinophils and basophils and promotes tissue eosinophilia.<sup>27-30</sup> In addition to this, IL-5 is secreted by Th2 cells, eosinophils and mast cells and promotes eosinophil activation and trafficking to the esophagus.<sup>10,27,31</sup> Both trials with anti-IL-5 and anti-IL-13 treatment in pediatric and adult EoE have demonstrated a reduction in esophageal eosinophilia.<sup>32-36</sup> Additionally, CPA-3 showed also a significant positive correlation between the absolute change in peak eosinophil counts at week 6 and the relative gene expression of CPA-3 after the diet. As such, a direct relationship between the density of eosinophils and mast cell markers (CPA-3) has been demonstrated both in our study and in previous reports.<sup>8,37</sup> These findings further support an important role for mast cell activation in EoE pathogenesis and in the mechanism of dietary treatment in reversing mast cell activity. Moreover, IL-13 also induces tissue remodeling (e.g., promoting collagen deposition), which leads to esophageal rigidity and fibrostenosis, resulting in clinical symptoms of dysphagia and food impactions.<sup>38-40</sup> This working mechanism may support our finding that IL-13 is expressed in significantly lower levels in patients with a clinical response after 6 weeks of elimination diet.

A significantly higher expression level of CAPN-14 and lower levels of DSG-1 was observed in patients with a "fibrotic" phenotype (vs. "non-fibrotic" phenotype) at baseline. Some data in the literature provide additional context for our findings. Increased expression of CAPN-14 is induced by IL-13, which leads to disruptive effects on the esophageal epithelium by impairment of barrier integrity in association with loss of DSG-1 expression.<sup>5,41-43</sup> A retrospective study by Lyle et al.<sup>44</sup> recently suggested CAPN-14 genetic variants being associated with earlier disease onset in pediatric EoE. In addition to this, longstanding eosinophilic inflammation is associated with esophageal remodeling and stricture formation.<sup>45</sup> CAPN-14 was found to be dynamically upregulated as a function of disease activity in previous studies.<sup>46</sup> Our findings of CAPN-14 being significantly more upregulated in "fibrotic" phenotypes, suggests that CAPN-14 may be linked to EoE patients with a more severe disease phenotype. In general, TGB- $\beta$  signaling pathway is considered as the central mediator of fibrosis in EoE.<sup>9,47</sup> Although visual changes in the esophagus may be seen on endoscopy as rings and strictures,

identification of sub epithelial fibrosis requires deep esophageal biopsies. This may be an explanation for the absence of a significant difference between transcripts of TGB- $\beta$  in these phenotypes.

Furthermore, only IL-10 (an anti-inflammatory cytokine) was expressed in significantly higher levels in patients presenting with a "food impaction" phenotype compared with the "non-food impaction" phenotypes prior to treatment. However, the reason for this remains unclear. Although data remains scarce on the role of IL-10 in EoE, higher levels of IL-10 expression between EoE and controls have been observed in a pediatric sample.<sup>13</sup> Since gene expression of IL-10 was significantly downregulated after the diet, the role of this anti-inflammatory cytokine may thus be related to an immunoregulatory response instead. In a pediatric EoE study by Rosenberg et al.,<sup>48</sup> it was observed that esophageal immunoglobulin (Ig) G4 levels correlated with eosinophils and levels of IL-10. Excess pro-inflammatory Th2 responses, as seen in clinical settings involving chronic antigen exposure (e.g., beekeepers) are known to induce regulatory T cells, which secrete high levels of IL-10, inducing class switching to IgG4. It has been suggested in previous literature that IgG4 production may be a compensatory mechanism to dampen the ongoing Th2 inflammatory response in EoE. Thus, our observations on IL-10 being significantly downregulated after the diet may be related to a reduction of food antigen exposure in the esophagus and a reduction in Th2 activation.

A few limitations of this study need to be acknowledged. First, this was a single-center study of a small sample of adults only, so it is difficult to compare results directly to prior gene expression studies that have been primarily performed in pediatric EoE populations. In addition, the small sample size is limiting its statistical power. Second, we did not include healthy individuals without EoE. We were therefore not able to assess whether expression levels normalized after diet treatment. Third, gene expression was only measured in biopsies taken from the mid-esophagus and were compared with peak eosinophil counts/hpf across different levels of the esophagus (distal, mid, and proximal). However, a study by Dellon et al.<sup>49</sup> showed that gene expression (RNA-later specimens) scores were similar across different levels (distal, mid, and proximal) of the esophagus and it is therefore not expected to have affected our results substantially. Fourth, the gene expression analysis was limited to 10 selected genes, so it is possible that additional differences might be observed after broader RNA sequencing. Finally, epithelial permeability changes are an important factor in EoE. Previous studies of our research group investigated the relationship between genes encoding for barrier integrity and permeability, including trans-epithelial electric resistance (TER), molecule flux in using chambers and intracellular spaces at electron microscopy.<sup>25,50</sup> Since these studies observed negative correlations for genes encoding for barrier integrity such as fillagrin and DSG-1 and TER, molecule flux and dilated intracellular spaces together with the fact that dilation of intracellular spaces on light microscopy is a less specific marker of permeability, these analysis were not performed in this current study. However, there are also multiple strengths that lend validity to the results. This is the first study evaluating the effect of an elimination diet on the expression

levels of pro-inflammatory and epithelial/barrier function related genes that were previously suggested to play an important role in EoE pathogenesis. Moreover, specimens were handled and stored uniformly, and extensive prospectively collected clinical data were available to allow full clinical, endoscopic, and histological characterization of all EoE patients. Another strength is the use of different clinical outcome measures (i.e., symptoms, endoscopic), and avoidance of observer bias by our blinded endoscopic scoring strategy.

In summary, this study suggests that elimination diets, in addition to a clinical and histological response, are associated with a broad transcriptional response at the level of the esophageal epithelium in EoE patients. Multiple pathways that are leading to this common disease state are affected after dietary treatment, with significant changes in gene expression markers related to inflammation (IL-5, IL-13, TSLP, POSTN CPA-3, CCL-26, and IL-10), epithelial/barrier function (DSG-1, CAPN-14) and remodeling/fibrosis (TGF- $\beta$ ). In particular, upregulation of CAPN-14 and lower levels of DSG-1 were associated with “fibrotic” phenotypes, whereas upregulation of IL-10 was linked to “food impaction” phenotypes. These results provide initial insight into genetic determinants of different presentations of EoE and provide a foundation for future mechanistic studies.

#### CONFLICT OF INTEREST

WErR, MAPD and MJW have no conflicts of interest. MTJvA is an employee of Danone Nutricia Research and salary is not dependent of outcomes of this study. BCAMvE is partly affiliated to Danone Nutricia Research. AJB has received research funding from Nutricia, SST, Norgine, Thelial and Bayer; speaker and/or consulting fees from Laborie, Reckitt Benckiser, Alimentiv, EsoCap, Medtronic, DrFalkPharma, Calypso biotech, Regeneron/Sanofi, Celgene, AstraZeneca and Arena.

#### AUTHOR CONTRIBUTIONS

WErR is the guarantor of the article. WErR, MJW, MAPD, and AJB were assisted in writing. WErR, MJW, and MTJvA contributed to conception and design. WErR, MAPD, BCAMvE, MTJvA, and AJB contributed to generation, collection, assembly, analysis, and/or interpretation of data. WErR, MJW, MAPD, BCAMvE, and AJB contributed to drafting of the article. WErR, MJW, MAPD, SRBME, MTJvA, BCAMvE, and AJB contributed to the approval of the final version of the manuscript.

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