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Lack of Association of the Esophageal Microbiome in Adults with Eosinophilic Esophagitis Compared with Non-Eosinophilic Esophagitis Controls

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ABSTRACT

Background & Aims: Changes in the esophageal microbiome have been reported in children with eosinophilic esophagitis (EoE), but few data exist for adults. We aimed to determine whether the esophageal microbiome differs in adults with and without EoE.

Methods: In a prospective cohort study, adults undergoing outpatient endoscopy were enrolled as incident EoE cases or non-EoE controls. Clinical, endoscopic, and histologic data were collected. An esophageal biopsy was utilized for microbiome analysis. Bacterial DNA was extracted and the V3-V4 region of the 16S rRNA gene was amplified and sequenced. Analyses were performed comparing microbiome features for cases and controls, and within cases for disease features, with correction for multiple hypothesis testing.

Results: A total of 24 incident EoE cases (mean age 40 years; 63% male; 100% white; 97 eos/hpf) and 25 controls (mean age 48, 36% male; 76% white; 1 eos/hpf) were analyzed. Principal coordinate analysis ordination failed to distinguish cases from controls. There were no microbiome differences within EoE cases based on clinical phenotype, presence of atopy, or endoscopic features. Use of proton pump inhibitors (PPIs), however, was significantly associated with 5 taxa including SR1 at the phylum level and *Burkholderia* at the genus level.

Conclusions: There were no significant differences in the esophageal microbiome between newly diagnosed EoE cases and non-EoE controls in adults, or within EoE cases based on clinical features. However, given the strong rationale for the esophageal microbiome in EoE pathogenesis, future studies should explicitly consider the presence of PPIs as a confounding feature.

Key words: eosinophilic esophagitis – microbiome – proton pump inhibitor – phenotype.

Abbreviations: EoE: eosinophilic esophagitis; eos/hpf: eosinophil per high-power field; EREFS: EoE Endoscopic Reference Score; FDR: false detection rate; GERD: gastroesophageal reflux disease; GI: gastrointestinal; H. pylori: Helicobacter pylori; OTU: operational taxonomical units; PPI-REE: proton pump inhibitor-responsive esophageal eosinophilia; PCoA: principal coordinate analysis; UNC: University of North Carolina.

INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic allergic/immune-mediated condition that may progress over time from an inflammatory to a fibrostenotic process [1, 2]. While incompletely understood, the pathogenesis involves a Th2 type cascade triggered by food or environmental allergens, likely in the setting of an esophageal epithelial barrier defect [3, 4]. The rapid rise in incidence and prevalence of EoE over the past 2-3 decades suggests environmental rather than genetic changes [5, 6], and a number of investigations have explored potential environmental risk factors including population density, climate, and season or aeroallergens [7-10]. More recently, there has been a focus on early life risk factors [11, 12]. Antibiotic administration in infancy, cesarean delivery, neonatal intensive care admission, absence of furred pets, and maternal infection have all been associated with a subsequent increased risk of developing EoE [13-17].

One common thread of these early life risk factors for EoE is that they might impact the gut microbiome [18]. This would be environmental exposures on the "micro" rather than "macro" level, and the altered microbiome has been demonstrated in a number of other non-EoE gastrointestinal conditions [19-21]. Additionally, *Helicobacter pylori* (*H. pylori*) infection has been reported as a risk factor for EoE, though this remains

controversial [22-26]. There have been two prior studies examining the role of the esophageal microbiome in EoE, both of which focused on pediatric patients. These reported selected differences in patients, predominantly children, with and without EoE, including an increase in *Haemophilus* in active EoE [27] and enrichment in the relative abundance of Proteobacteria in EoE [28]. However, these data remain to be confirmed, and there are scant data on the role of the esophageal microbiome in adults with EoE.

Therefore, the aims of this study were to determine whether the esophageal microbiome differs between adult EoE cases and non-EoE controls, and to examine whether different clinical phenotypes impact the observed microbiome within EoE cases. Based on the prior reports in children, we hypothesized that similar differences in adults would be observed.

METHODS

This study was a secondary analysis of data and biospecimens collected during a prospective cohort study and was approved by the UNC IRB (#15-0163). Details of the parent study have been previously described [29-33], and subjects who participated provided informed consent for future use of banked biopsy samples. In brief, adults (age 18-80) who were undergoing a clinically indicated upper endoscopy for upper gastrointestinal (GI) symptoms (dysphagia, heartburn, chest pain or abdominal pain) were enrolled. Subjects were excluded if they had known EoE or other eosinophilic GI disorders, active GI bleeding, known esophageal varices, known esophageal cancer, or prior esophageal surgery. Full demographic and clinical details were captured on standardized case-report forms. During endoscopy, all findings were recorded, including typical features of EoE which were classified using the EoE Endoscopy Reference Score (EREFS) system [34, 35]. Both clinical and research biopsies were obtained, and peak eosinophil counts (eosinophils per high-power field [eos/hpf]; hpf = 0.24mm^2) were quantified using our previously validated protocol [36-38]. After all data were received, patients were classified as an incident EoE case if they met consensus diagnostic criteria at the time of the study design [1, 39]. These diagnostic criteria excluded patients who were previously termed proton pump inhibitor-refractory esophageal eosinophilia (PPI-REE) but who would now be classified as EoE [40], so only PPI non-responsive EoE cases were included. EoE cases were otherwise not on anti-inflammatory treatment (swallowed topical corticosteroids, dietary elimination, biologics). Controls were subjects with either heartburn or dysphagia-predominant symptoms, regardless of the etiology of their symptoms or whether they were on treatment for symptoms, which did not meet EoE diagnostic criteria. Cases and controls were defined in this fashion, so they were reflective of subjects undergoing upper endoscopy for GI symptoms.

For a sample collection, a single mid-esophageal biopsy, taken during the study endoscopy for future research purposes (which was at the time of EoE diagnosis for the cases), was immediately flash-frozen in liquid nitrogen and stored at -80oC, with no subsequent thaw-freeze cycles. After patient recruitment and sample collection for the parent study was complete, these samples were retrieved and used for the following microbiome analysis. The rationale for using the mid-esophageal biopsy was to avoid potential acid exposure that might impact a distal esophageal biopsy and to minimize oral microbiome that might be seen with a proximal biopsy.

Bacterial DNA was extracted using standard techniques, followed by a polymerase chain reaction (PCR) amplification of the V3-V4 region of the bacterial 16S rRNA gene and sequencing on the Ion Torrent platform [41, 42]. Sequences were processed through Qiime 1.9 for phylogenetic analysis and taxonomic identification [43]. Qiime 1.9 was used in lieu of Qiime 2 due to optimization of Qiime 2 for the Illumina platform. The average sequence length was 300 bp and average read depth was 84,000 reads.

Bioinformatics analysis began with the use of BioLockJ (https://github.com/BioLockJ-Dev-Team/BioLockJ) to demultiplex sequence data into individual fastq files. Trimming consisted of the removal of barcode and linker primers from samples reads and conversion to fasta format. Assignment of reads to Operational taxonomical units (OTUs) relied on Qiime, version 1.9, to a closed reference database at 97% threshold. Taxonomical classification of assigned OTUs was based upon the Silva database, version 128. Additional classification of reads against the Silva database were employed through DADA2 and the RDP classifier [44, 45].

Statistical analysis of classified reads started with beta diversity using Bray Curtis dissimilarity matrices in R (http://www.R-project.org/; package Vegan: https://cran.r-project.org/web/packages/vegan/vegan.pdf). Variables of interest were EoE case status, gender, atopy, PPI use, endoscopic findings, and fibrostenotic and inflammatory endoscopic phenotypes. Analysis of individual taxa at phylum and genus were correlated with variables of interest using univariate linear models. Benjamini Hochberg correction was applied with less than 10% established as the significance level.46 Power analysis was averaged over 1000 trials for both phylum and genus and corrected by Benjamini Hochberg (github.com/afodor/metagenomicsTools/blob/master/src/powerSims/powerSimulations.txt).

RESULTS

Samples were available for analysis from 24 EoE cases and 25 non-EoE controls. Compared to controls, cases tended to be somewhat younger (mean age 40 vs 48 years; p=0.1), male (63% vs 36%; p=0.06), and white (100% vs 76%; p=0.01) (Table I). Dysphagia was more common in cases (100% vs 80%; p=0.02) while heartburn was more common in controls (48% vs 0%; p<0.001), as expected by the study design and inclusion criteria. Typical EoE endoscopic findings of exudates, rings, edema, furrows, and strictures were more common in cases, with higher mean EREFS scores (3.9 vs 0.2; p<0.001). The baseline peak eosinophil count was 96.9 eos/hpf in cases and 0.5 in controls. For the control group, the final diagnosis was gastroesophageal reflux disease (GERD) in 12, esophageal dysmotility in 6, and a functional GI disorder in 7.

To test the hypothesis that EoE was associated with members of the microbial community, we performed Ion Torrent sequencing. Classification with the Qiime algorithm to the genus level with the Silva database resulted in 56 non-

Table I. Baseline clinical, endoscopic, and histologic characteristics of the study population

	Controls (n = 25)	EoE cases (n = 24)	p*
Age (mean years ± SD)	47.6 ± 16.4	40.3 ± 13.6	0.10
Male (n, %)	9 (36)	15 (63)	0.06
White (n, %)	19 (76)	24 (100)	0.01
Symptoms (n, %)			
Dysphagia	20 (80)	24 (100)	0.02
Heartburn	12 (48)	0 (0)	< 0.001
Abdominal pain	1 (4)	0 (0)	0.32
Nausea/Vomiting	0 (0)	0 (0)	--
Any atopic disorder (n, %; n = 35)	16 (64)	19 (79)	0.24
Asthma	5 (20)	7 (29)	0.46
Atopic dermatitis	1 (4)	2 (8)	0.53
Allergic rhinitis/sinusitis	16 (64)	17 (71)	0.61
Food allergies	2 (8)	6 (25)	0.11
EGD findings (n, %)			
Normal	7 (28)	1 (4)	0.02
Rings	1 (4)	21 (88)	< 0.001
Strictures	2 (8)	11 (46)	0.003
Narrowing	1 (4)	5 (21)	0.07
Crepe paper mucosa	0 (0)	1 (4)	0.30
Furrows	1 (4)	22 (92)	< 0.001
White plaques/exudates	0 (0)	14 (58)	< 0.001
Edema/decreased vascularity	0 (0)	10 (42)	< 0.001
Hiatal hernia	11 (44)	3 (13)	0.02
Dilation performed	5 (20)	11 (46)	0.05
Total EREFS score	0.2 ± 0.5	3.9 ± 2.0	< 0.001
Baseline max eosinophil count (mean ± SD)	0.5 ± 1.4	96.9 ± 60.0	< 0.001
Other histologic findings (n, %)			
Eosinophil degranulation (n = 27)	0 (0)	14 (93)	< 0.001
Eosinophil microabscesses (n = 26)	0 (0)	13 (93)	< 0.001
Basal layer hyperplasia (n = 24)	1 (8)	9 (75)	0.001
Spongiosis (n = 35)	7 (41)	17 (94)	0.001
Adequate lamina propria (n = 28)	5 (33)	1 (85)	0.006
Lamina propria fibrosis (n = 9)	0 (0)	6 (86)	0.02

*means compared with *t*-tests and proportions compared with chi-square; EoE: eosinophilic esophagitis; EREFS: EoE Endoscopy Reference Score; EGD: esophagogastroduodenoscopy; SD: standard deviation.

rare taxa present in at least 25% of all samples at the genus level. Principal coordinate analysis (PCoA) ordination at the phyla (Fig. 1A) and genus (Fig. 1B) level failed to distinguish case from control samples. Likewise, when we considered the log-normalized relative abundance of each taxa individually, we also found no significant differences at a 10% FDR by *t*-test at the phyla (Fig. 2, Supplementary file Table I) or genus level (Supplementary file Table II).

A more complex linear model that took into account gender, race, and case/control status also did not find any differences at a 10% FDR for any covariates at either the phylum or genus levels after multiple hypothesis correction (data not shown). At an uncorrected threshold of p <0.05, 2 taxa (*Aggregatibacter* and Burkholderia) were significantly different from this model between cases and controls, but neither taxa survived FDR correction. There was a similar lack of significant associations with case control status using the DADA 2 and RDP pipelines (data not shown). Post-hoc power simulations at a threshold of 80% power suggest that at a 10% FDR cutoff a difference between case and control would be detectable at an effect size greater than approximately 1.0 with our study sample size.

Additional univariate t-tests examined the relation between clinical features and esophageal phenotypes and how they influenced the esophagus microbiome (Supplementary file Tables III and IV). At both the phylum and genus levels in the overall population of case and control, the presence/absence of any atopic disease (as well as individual conditions such as food allergies or allergic rhinitis) were not significantly

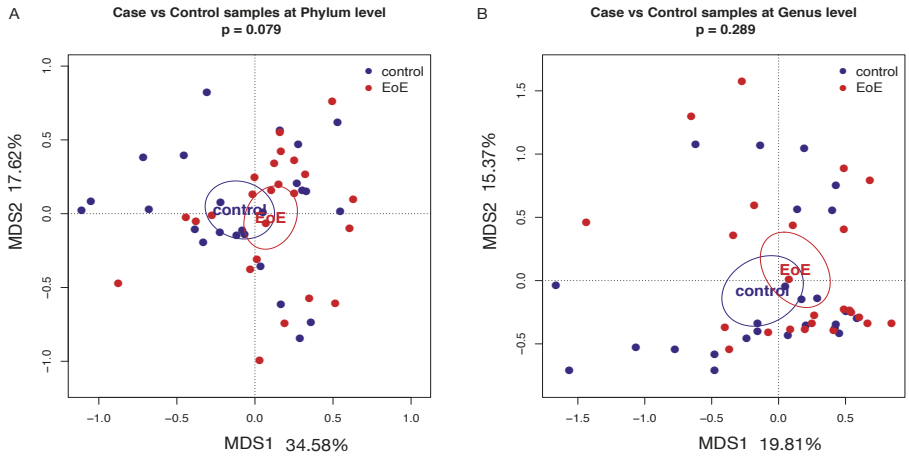

Fig. 1. Overall Principal Coordinate Analysis (PCoA) of Beta Diversity in the EOE cohort at both the phylum (A) and genus (B) levels.

associated with any of the non-rare taxa at a 5% FDR (data not shown). Within the EoE patients, there was also a lack of a significant correlation between any of the non-rare taxa and the presence of endoscopic findings including exudates, rings, edema, furrows, strictures, or esophageal dilation. There were also no correlations based on inflammatory or fibrostenotic endoscopic phenotypes in general, or by histologic features. Construction of Bray Curtis dissimilarity matrices and dimensionality reduction through principal coordinate analysis did not identify differences in microbiome beta diversity for any of the previously mentioned variables (data not shown).

We also examined the impact of PPIs on the microbiome. While there was no overall shift in microbiome diversity seen by PCoA ordination (Fig. 3), there were 5 taxa across phylogenetic levels that were significantly associated with patients taking PPIs even after conservative multiple hypothesis correction of 5% BH FDR (Fig. 4 and Supplementary file Fig. 1).

Examination of the distribution of all p-values suggested that for most taxa, PPIs were not associated, but there was one additional taxa *Methylobacterium* (Fig. 4C), that was distinct from the expectations under the null hypothesis, although this taxa was not significant after accounting for multiple hypothesis testing. In all these cases, PPIs increased the abundance of taxa from near-zero (Fig. 4). The number of individuals on PPIs was 40 and the number of individuals not on PPI was 9 and all 9 of these individuals were non-EoE controls. This prevented further exploration of additional clinical features and PPI use. We did perform a sub-analysis of the microbiome by PPI use within only the controls (n=16 on PPIs; n=9 not on PPIs) and did not see any significant associations (data not shown), though this was likely from the lack of power related to the small sample size for this sub-analysis.

DISCUSSION

Early life risk factors have been identified for EoE, including the use of antibiotics during infancy, cesarean delivery, neonatal intensive care admission, that have the potential to alter the

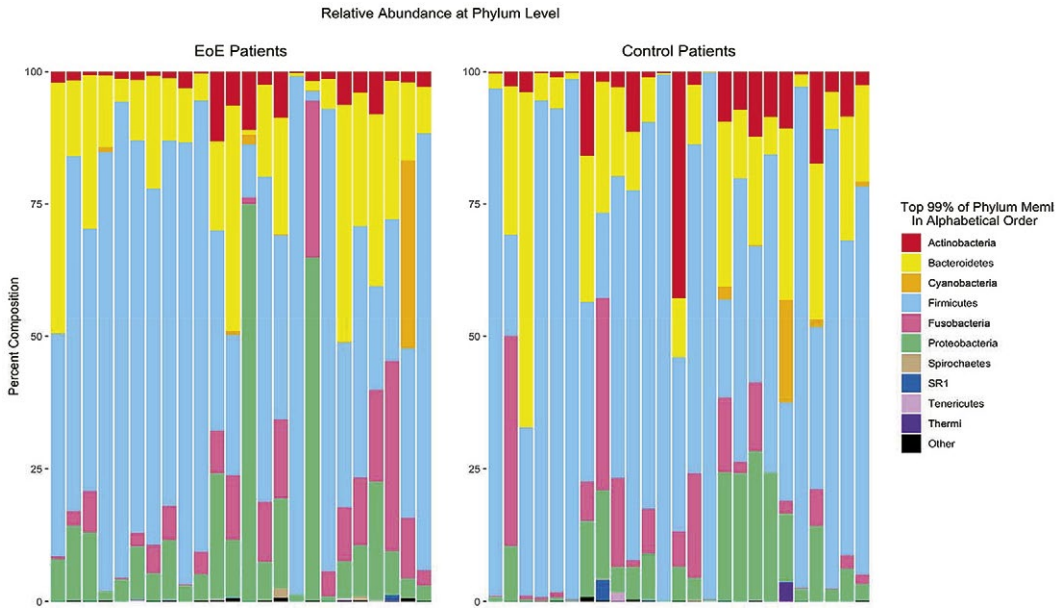

Fig. 2. Phylum Level Esophagus Composition for both eosinophilic esophagitis (EoE) patients and Non-EoE patients labeled as controls.

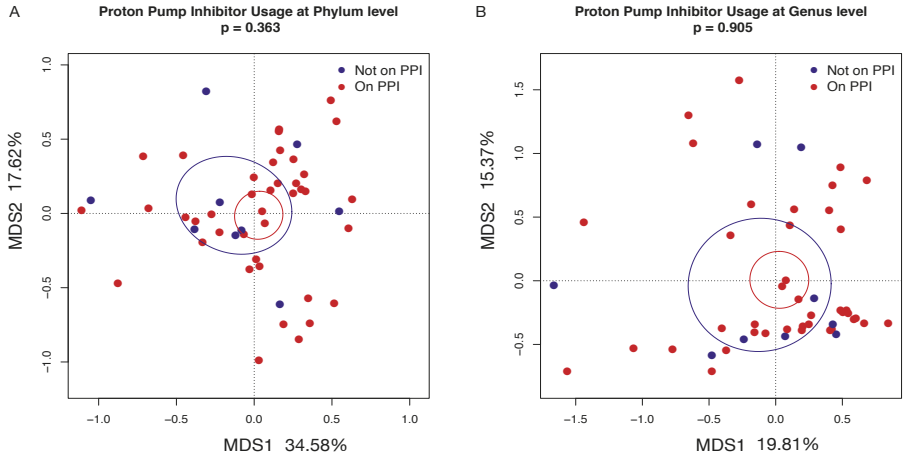

Fig. 3. Principal Coordinate Analysis (PCoA) of Beta Diversity in the EOE cohort for patients using PPI drugs at both the phylum (A) and genus (B) levels.

microbiome and perhaps impact EoE pathogenesis [12, 14]. The esophageal microbiome has not been extensively explored in adults with EoE, and we used prospectively banked esophageal biopsies from a cohort of well-characterized EoE cases (with an incident diagnosis) and non-EoE controls to address this issue. Contrary to our hypothesis that there would be differences in the esophageal microbiome, our results suggested the microbial community has a small to no effect on EoE in adults at the time of diagnosis. At an uncorrected threshold of $p<0.05$, there were a few small differences between non-EoE and EoE patients at the genus level, but no significant differences were observed at the phylum level or at either phylum or genus using a threshold that corrects for multiple hypothesis testing. Moreover, there were no significant differences in the esophageal microbiome of EoE cases based on clinical phenotype, including presence of atopy, a fibrostenotic vs. inflammatory phenotype, or by histologic features.

The esophagus is a relatively new area of study in the human microbiome field [21], with initial work highlighting that distal esophageal biopsies from normal patients were predominated by the *Streptococcus* genus, while gram-negative anaerobes or microaerophiles were seeing in patients with

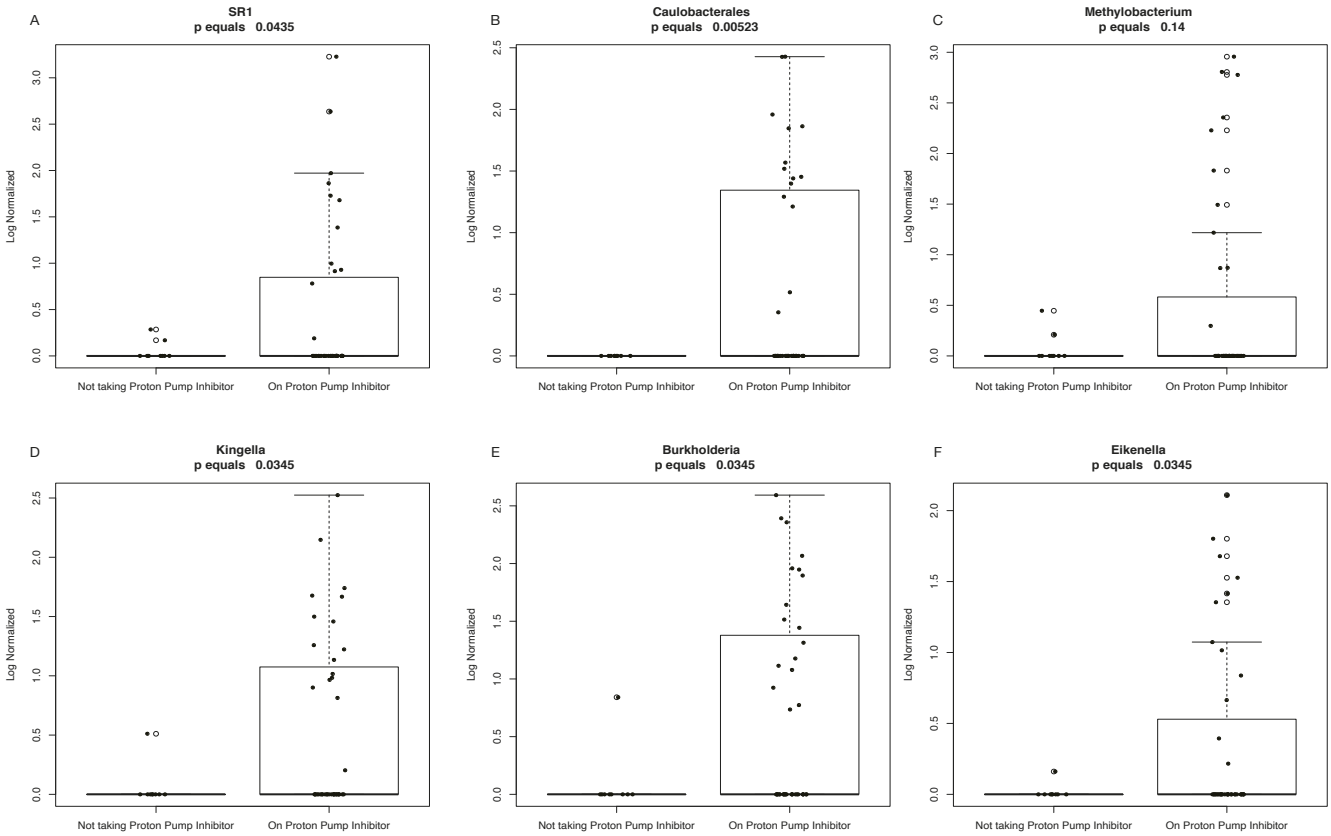

Fig. 4. Distributions of LogNormalized Read Counts for Statistically Significant Taxa at (A) Phylum level SR1 (B) Order level (D,E,F) Genus Level. (C) Genus Member who Deviated in QQplot (Suppl. Fig 1) but was not statistically significant after FDR correction.

reflux esophagitis or Barrett's esophagus [47]. A related study that used the esophageal string test, a minimally invasive sampling device, similarly noted that the most common genera were Streptococcus, *Prevotella*, and *Veillonella* [48]. The first study of the microbiome in EoE was by Harris et al. [27], again using the esophageal string to sample the esophagus. After analyzing specimens from 11 active EoE patients, 26 with inactive EoE, 8 GERD controls, and 25 normal controls (a combination of pediatric and adult patients), they found that *Haemophilus* was increased in active EoE compared with normal controls, as was the bacterial load but not diversity. In a study by Benitez et al. [28], 18 children with active EoE and 15 with inactive disease were also found to have differences, with enrichment in the relative abundance of Proteobacteria (*Neisseria* and *Corynebacterium*) in those with active EoE, and enrichment in *Streptococcus* and *Atopobium* genera in non-EoE controls. In patients with longitudinal samples available, dietary interventions did not lead to global microbiome changes, though some food re-introductions led to enrichment in *Granulicatella* and *Campylobacter* genera. More recently, there has also been an investigation of the stool microbiome [49], as well as a study of the salivary microbiome [50], in EoE. There is strong rationale for the role of the microbiome in EoE. In addition to the potential early life exposures already discussed in EoE and other atopic diseases [12, 19], there have been recent investigations assessing the role of toll-like receptors in EoE [51-53]. In addition to being key mediators between microbiota and epithelial surfaces in the esophagus and gut, these pathways appear to activate EoE inflammation and impact barrier function in the esophagus as well [51, 53]. However, it is unknown whether in observed changes in the esophagus, the microbiome is a cause of EoE or an effect of structural and inflammatory changes that are induced by EoE. It is also possible that these early life factors impact the microbiome in the gut and this leads to changes in immune development.

Our findings contrast with the prior Harris et al. [27] and Benitez et al. [28] data in EoE. We neither observed changes in *Haemophilus* nor replicated the strong enrichment of Proteobacteria members associated with EoE. Further, we did not see an association between microbiome and EoE disease features. Several potential reasons merit consideration. First, it is possible that our control subjects were not appropriate. These were not truly "healthy" controls, in that they were undergoing upper endoscopy for evaluation of significant clinical symptoms, had diagnoses of GERD, esophageal dysmotility, and functional GI disorders, had hiatal hernias, and atopic conditions. Because these underlying conditions might also lead to microbiome alterations, it could potentially bias the results towards the null. However, this is similar to the control definitions in prior studies. Second, we could be assessing the wrong time point in the course of EoE. Though we assessed newly diagnosed incident EoE cases, patients typically have a long symptom duration prior to diagnosis [54] and our samples were not obtained at disease onset. A pediatric population may well be closer to disease onset, in a time period of immune development in which microbiome is critical to normal structure and function. Third, our sample size could be too small or there could be too much inter-subject variation. In that case, longitudinal studies within individuals may be more revealing. Our *post-hoc* power calculations suggest we might be underpowered for identifying individual taxa where thresholds of significance require correction for multiple hypothesis testing, and this is a study limitation. However, we also did not detect any difference between cases and controls in the microbial community using PCoA ordination, which does not require correction for multiple hypothesis testing. Fourth, all of our EoE cases had been treated with PPI in order to confirm the disease diagnosis, as required by consensus diagnostic guidelines at the time. PPIs are known to have an effect on the microbiome [55-57], but a large proportion (two-thirds) of the controls were also on PPIs at the time of sample collection, thus making a PPI effect less likely. However, future studies of the esophageal microbiome would ideally be conducted in PPI-naïve subjects. Fifth, while unlikely, there could have been unreported antibiotic use. Last, there could be a biopsy sampling issue. We selected a mid-esophageal biopsy to avoid potential contamination by either oral flora proximally or gastric refluxate distally. In contrast, the Harris study [27] used a string that was able to sample the entire esophagus. This technique also collected luminal bacteria while our biopsies were limited to adherent bacteria. More esophageal samples (rather than the single biopsy we used) could be more reflective of the overall esophageal microbiome or could elucidate differences in microbiome along the esophageal axis, and these issues will be a focus of future research. Despite these potential limitations, our study also has a number of strengths. It utilized prospective data from newly diagnosed EoE cases in a rigorous study design, had comprehensive standardized data collection, specimen handling, and storage, and highly detailed patient characterization. It also utilized state-of-the-art bioinformatic analysis strategies, with appropriate correction for multiple testing.

Future studies should explicitly compare adult to pediatric patients and consider the impact of antibiotic treatment as well as PPI use. These measures will be required in order to resolve the differences between our study and previous work. Because all the studies to date – including ours – have modest sample sizes and given the apparent small effect size of associations with EoE, larger cohorts may ultimately be required to see consistent results. In addition, whole-genome sequencing may yield functional differences that may have a larger effects size than was observed with the 16S sequencing we performed here. Ideally, if samples can be obtained from very early on in the disease course, then we may be able to make inferences about causality rather than associations related to EoE pathogenesis.

CONCLUSIONS

We did not find significant differences in the esophageal microbiome as determined from esophageal biopsy samples between newly diagnosed EoE cases and non-EoE controls in adults. We also did not observe differences within EoE cases based on clinical, endoscopic, or histologic disease features. However, given the strong rationale for the potential role of the esophageal microbiome in EoE pathogenesis, a future study remains warranted.

Conflicts of interest: None to declare.

Authors' contribution: E.S.D., T.O.K. conceived the project and designed the study. E.S.D obtained funding. E.S.D., A.N.MC. collected the data. J.J., E.S.D., A.A.F drafted the manuscript. J.J., E.S.D., A.N.MC., S.S., E.T.J., A.A.F., T.O.K analyzed and interpreted the data and critically revised the manuscript.

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