

Pediatric eosinophilic esophagitis is associated with changes in esophageal microRNAs

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Submitted 2 April 2014; accepted in final form 13 August 2014

Zahm AM, Menard-Katcher C, Benitez AJ, Tsoucas DM, Guen CL, Hand NJ, Friedman JR. Pediatric eosinophilic esophagitis is associated with changes in esophageal microRNAs. *Am J Physiol Gastrointest Liver Physiol* 307: G803–G812, 2014. First published August 21, 2014; doi:10.1152/ajpgi.00121.2014.—The incidence of eosinophilic esophagitis (EoE) has increased in the past several years, yet our understanding of its pathogenesis remains limited. To test the hypothesis that microRNAs (miRNAs) are altered in children with EoE, miRNAs were profiled in esophageal mucosa biopsies obtained from patients with active disease ($n = 5$) and healthy control subjects ($n = 6$). Fourteen miRNAs were significantly altered between groups; four of these miRNAs were decreased in EoE patients. A panel of five miRNAs (miR-203, miR-375, miR-21, miR-223, and miR-142-3p) were selected for validation in an independent set of samples from control ($n = 22$), active disease ($n = 22$), inactive disease ($n = 22$), and gastroesophageal reflux disease ($n = 6$) patients. Each panel miRNA was significantly altered among groups. miRNA changes in esophageal biopsies were not reflected in the circulating RNA pool, as no differences in panel miRNA levels were observed in sera collected from the four patient groups. In addition, in contrast to previous studies, no change in esophageal miRNA levels was detected following treatment that resolved esophageal eosinophilia. In an effort to identify the ramifications of reduced esophageal miR-203, miR-203 activity was inhibited in cultured epithelial cells via expression of a tough decoy miRNA inhibitor. Luciferase reporter assays demonstrated that miR-203 does not directly regulate human IL-15 through targeting of the IL-15 3'-untranslated region. From these experiments, it is concluded that miRNAs are perturbed in the esophageal mucosa, but not the serum, of pediatric EoE patients. Further investigation is required to decipher pathologically relevant consequences of miRNA perturbation in this context.

eosinophilic esophagitis; microRNA; serum microRNA; circulating microRNA; atopic disease

EOSINOPHILIC ESOPHAGITIS (EoE) is a chronic inflammatory disease characterized by infiltration of eosinophils into the esophageal mucosa in response to allergen exposure. Other prominent histological features of EoE include elevated levels of B and T cells, activated mast cells, and basal cell hyperplasia (1, 3, 43, 45). In young children, EoE can lead to feeding refusal/intolerance and failure to thrive, while older children often complain of chronic dysphagia and food impaction. Other complications include esophageal stricture and spontaneous perforation. Genome-wide association analyses have identified EoE risk variants, most notably, mapping to regions that include thymic stromal lymphopoietin, a cytokine elevated in

EoE, and CAPN14, a protein-coding gene expressed in the esophagus and upregulated by IL-13 (20, 33, 38). However, despite a rapid increase in EoE diagnoses in children and adults, the instigating and perpetuating mechanisms of the disease remain largely unknown (9). Differentiated epithelial cells of the gastrointestinal tract facilitate cross talk with the external environment by regulating local innate and adaptive immune cells (31, 37, 54). Thus, changes in the architecture and differentiation status of the esophageal epithelium associated with EoE are likely to perturb local homeostatic immune signaling.

MicroRNAs (miRNAs) are regulatory RNAs that function to destabilize target mRNAs by recruiting RNA-induced silencing complex machinery. miRNAs are potent modulators of inflammatory signaling cascades (19, 34, 47) and are necessary for homeostatic maintenance in the gastrointestinal tract (5, 28). Changes in esophageal miRNAs have been observed in EoE and other esophageal pathologies (11, 24, 26, 51). Using a quantitative RT-PCR (qRT-PCR)-based platform, T. X. Lu et al. (26) profiled miRNAs in human esophageal biopsies from healthy subjects and pediatric EoE patients. They identified 21 upregulated and 11 downregulated miRNAs in the EoE sample group. Subsequently, S. Lu et al. (24) found six esophageal miRNAs to be altered in pediatric EoE patients. Yet virtually nothing is known regarding the consequences of altered miRNA regulation in these contexts. In addition, T. X. Lu et al. reported altered circulating miRNAs in EoE, raising the possibility of a novel, noninvasive biomarker.

The goals of this study were to assess mucosal miRNA abundance in the esophagus of pediatric EoE patients and healthy controls and to independently validate the miRNA changes reported in previous studies. Esophageal miRNAs were profiled in pediatric EoE patients and healthy controls, and the results were confirmed in large, independent sample sets. Some of the most abundant esophageal miRNAs were perturbed in EoE patients. However, changes in esophageal miRNA levels were not reflected in the circulating miRNA fraction. Longitudinal analyses found that esophageal miRNA levels did not track with improved eosinophil counts following medical and dietary therapy. Luciferase reporter assays suggested that miR-203 does not regulate human IL-15, despite the presence of a predicted miR-203 binding site within its 3'-untranslated region (UTR).

MATERIALS AND METHODS

Patient samples. Each patient or parent/guardian provided informed consent to participate in the study. Esophageal biopsies and/or blood samples were obtained during upper endoscopy as part of an Institutional Review Board-approved study at the Children's Hospital

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of Philadelphia Research Institute. Control samples were obtained from patients whose endoscopy and histology results were normal, despite upper gastrointestinal symptoms. EoE and gastroesophageal reflux disease (GERD) diagnoses were determined by the treating physician according to clinical, endoscopic, and histological findings. Active EoE was defined by a peak eosinophil count of ≥ 15 per high-power field (hpf), along with symptoms of esophageal dysfunction and a lack of response to ≥ 6 wk of proton pump inhibitor treatment (13, 23). Inactive EoE patients were those who were previously diagnosed with active EoE but had < 15 eosinophils per hpf at the time of biopsy. GERD patients were defined as having symptoms consistent with reflux esophagitis but < 10 eosinophils per hpf on biopsy. Patient demographic and clinical information is provided in Tables 1 and 2. Esophageal biopsies collected during endoscopy were immediately flash-frozen and stored at -80°C until they were processed. Blood samples were stored at room temperature for 30 min, centrifuged twice sequentially at 855 g for 10 min each to remove blood cells, and stored at -80°C until they were processed.

RNA isolation. Flash-frozen esophageal biopsies were pulverized in microcentrifuge tubes on dry ice using a chilled pestle and immediately processed. Total RNA from esophageal tissue or 60 μl of serum was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions. During serum RNA isolation, the exogenous *Caenorhabditis elegans* miRNAs cel-miR-54 and cel-miR-238 [22.5 and 15 pM, respectively, added as normalizing controls (30)] and MS2 RNA [3 μg , used to increase RNA recovery (2)] were added immediately following serum denaturation. For serum samples used in the profiling experiment, 3 μl of a spike solution containing exogenous miR-302a, miR-302d, and miR-372 (5, 5, and

10 pM, respectively) were added as positive controls. RNA samples were eluted in a volume of 100 μl .

miRNA profiling. Tissue miRNA levels were profiled using the nCounter human version 1 miRNA expression assay kit (nanoString Technologies, Seattle, WA). Data were processed after removal of all counts of miRNAs measured by the nCounter human version 1 kit that are no longer recognized by miRBase release 20 (15, 53). Individual miRNA counts were normalized to the total counts for each sample. Serum miRNA levels were profiled using the TaqMan Low Density Array Human MicroRNA Panels A and B version 2.0 (Life Technologies, Grand Island, NY). Three microliters of total serum RNA were used as input in a 7.5- μl RT reaction. Twelve cycles of cDNA preamplification were then performed using 2.5 μl of the RT reaction product as input according to the manufacturer's instructions. Serum miRNA array data were normalized using the median threshold cycle (C_t) of each array, and individual miRNA levels were calculated as $2^{(C_{t,\text{median}} - C_{t,\text{miRNA}})}$.

Quantitative RT-PCR. RT was performed using the TaqMan MicroRNA RT and miRNA assay kits (Life Technologies) with 33.3 ng of tissue RNA or 3.33 μl of eluted serum RNA as input. Amplification was performed in duplicate using TaqMan Universal PCR Master Mix and miRNA assay kits (Life Technologies). Tissue miRNA data were normalized to the mean C_t of RNU44 and RNU48 levels. Serum miRNA data were normalized to the mean C_t of cel-miR-54 and cel-miR-238. miRNA levels were calculated as $2^{(C_{t,\text{median}} - C_{t,\text{miRNA}})}$. Interplate calibration was performed with four calibrator samples using the method of Hellemans et al. (17).

Cell culture and luciferase assays. A 601-bp fragment containing 492 bp of the human IL-15 3'-UTR was amplified from IMAGE

Table 1. Characteristics of patients in case-control experiments

	EoE			
	Active	Inactive	Healthy	GERD
<i>Serum subjects</i>				
<i>n</i>	17	17	10	12
Eos/hpf	42.5 ± 15.6	2.2 ± 2.2*	Not detected	3.7 ± 2.9*
Age, yr	9.7 ± 4.8	9.1 ± 5.0	8.5 ± 6.1	10.1 ± 5.9
Sex, male, <i>n</i> (%)	16 (90)	16 (90)	9 (90)	10 (80)
Race, white, <i>n</i> (%)	16 (90)	16 (90)	9 (90)	11 (90)
History of atopy, <i>n</i> (%)	16 (90)	16 (90)	4 (40)	6 (50)
Asthma	10 (60)	10 (60)	1 (10)	5 (40)
Atopic rhinitis	9 (50)	10 (60)	1 (10)	2 (20)
Atopic dermatitis	9 (50)	5 (30)	2 (20)	4 (30)
Age at EoE diagnosis, yr	7.9 ± 6.0	6.2 ± 5.1		
EoE treatment, <i>n</i> (%)				
None	6 (35)	0 (0)*		
Diet	10 (59)	16 (94)*		
STS	1 (6)	0 (0)		
Diet and STS	0 (0)	1 (6)		
<i>Tissue subjects</i>				
<i>N</i>	27	22	28	6
Eos/hpf	39.1 ± 15.7	2.2 ± 3.3*	Not detected	3.8 ± 2.1*
Age, yr	10.8 ± 4.6	8.6 ± 4.8	9.6 ± 5.2	8.6 ± 7.1
Sex, male, <i>n</i> (%)	20 (91)	20 (91)	24 (86)	6 (100)
Race, white, <i>n</i> (%)	20 (91)	20 (91)	26 (93)	5 (83)
History of atopy, <i>n</i> (%)	19 (86)	19 (86)	19 (68)	3 (50)
Asthma	8 (36)	13 (59)	13 (46)	3 (50)
Atopic rhinitis	12 (55)	10 (45)	2 (7)	2 (33)
Atopic dermatitis	15 (68)	7 (32)	6 (21)	1 (17)
Age at EoE diagnosis, yr	9.4 ± 5.3	6.2 ± 4.9		
EoE treatment, <i>n</i> (%)				
None	17 (63)	0 (0)*		
Diet	7 (26)	20 (91)*		
STS	2 (7)	2 (9)		

Values for continuous variables are means $\pm$ SD. EoE, eosinophilic esophagitis; GERD, gastroesophageal reflux disease; Eos, eosinophils; hpf, high-power field; STS, swallowed topical steroids. No statistical difference by analysis of variance between groups for age, sex, race, or atopy, unless otherwise indicated.

* $P < 0.05$ vs. active EoE.

Table 2. Characteristics of patients in longitudinal experiments

	Responding (n = 12)	Nonresponding (n = 12)
Age at 1st biopsy, yr	11.7 ± 4.5	8.5 ± 5.1
Age at diagnosis, yr	10.0 ± 4.6	6.6 ± 6.3
Sex, male, %	91.7	75.0
Race, white, %	50.0*	75.0
History of atopy, %	100.0	83.3
Asthma, %	100.0*	58.3
Allergic rhinitis, %	58.3	41.7
Atopic dermatitis, %	66.7	50.0
Eos/hpf		
1st biopsy	37.7 ± 22.3	32.1 ± 15.1
2nd biopsy	2.9 ± 4.3	53.0 ± 32.9
Time between biopsies, wk	33.8 ± 25.0	28.0 ± 20.4
Intervention between biopsies, n (%)		
Elemental diet	1 (8)	1 (8)
Elimination diet		
Multiple foods	1 (8)	3 (25)
Single food	1 (8)	5 (33)
STS		
OVB	6 (50)	0 (0)
Fluticasone	2 (17)	1 (8)
Diet and STS	1 (8)	2 (17)
Proton pump inhibitor	0 (0)	1 (8)

Values for continuous variables are means ± SD. OVB, oral viscous budesonide. **P* < 0.05 vs. nonresponding (by χ^2 test).

consortium cDNA clone 40044736 (Thermo Fisher Scientific, Waltham, MA) and subcloned into pMiRcheck2 (16). The miR-203 sensor plasmid was constructed by replacement of the *XhoI*-*NotI* fragment of pMiRcheck2 with a double-stranded oligonucleotide containing two miR-203 target sequences (miR-203 reverse complement, with a central bulged mismatch) separated by a five-nucleotide spacer. A tough decoy ("TuD" miRNA inhibitor) expression vector platform "pEN TuDparent," analogous to that described by Xie et al. (50), was generated by ligation of a double-stranded oligonucleotide (hU6TuD-top/hU6TuD-bot) into *NdeI*-*XbaI*-digested pEN_hu6 miRc2 (41). pEN_TuDctrl and pEN_TuD203 were generated by replacement of the *BfuAI* fragment in pEN TuDparent with double-stranded oligonucleotides containing scrambled miRNA binding sites or sites that act as decoys for miR-203. The resulting plasmids constitutively express TuD hairpins from the RNA polymerase III-dependent human U6 promoter. Oligonucleotides utilized in this study are listed in Table 3. MCF-7 human breast adenocarcinoma epithelial cells (American

Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. For luciferase reporter experiments, 5.0×10^4 cells were seeded in 24-well plates and transfected 24 h later with 900 ng of expression vector and 10 ng of luciferase reporter using FuGENE HD transfection reagent (Promega, Madison, WI) in the absence of antibiotics. Lysates were collected 24 h after transfection and assayed for *Renilla* and firefly luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analyses. Expression profiling data sets from the nCounter and TaqMan low-density array experiments were analyzed using Statistical Analysis of Microarrays (SAM) software (Stanford University, Stanford, CA) (44). All other statistical analyses were performed using Stata 11.0 (StataCorp, College Station, TX). The correlation heat map was created using Matrix2png (35). Hierarchical cluster analysis was performed using complete linkage with Euclidean distance for all esophageal miRNAs detected above background in all samples. Differences in miRNA levels were determined using Kruskal-Wallis one-way analysis of variance by ranks. To control for multiple testing, post hoc comparisons were performed using Holm-Bonferroni-corrected *P* values. Correlations among variables were determined using the Spearman rank-order correlation test with Bonferroni-corrected *P* values. Longitudinal changes in miRNA levels were determined using multilevel mixed-effects linear regression. The critical significance level α was defined as 0.05.

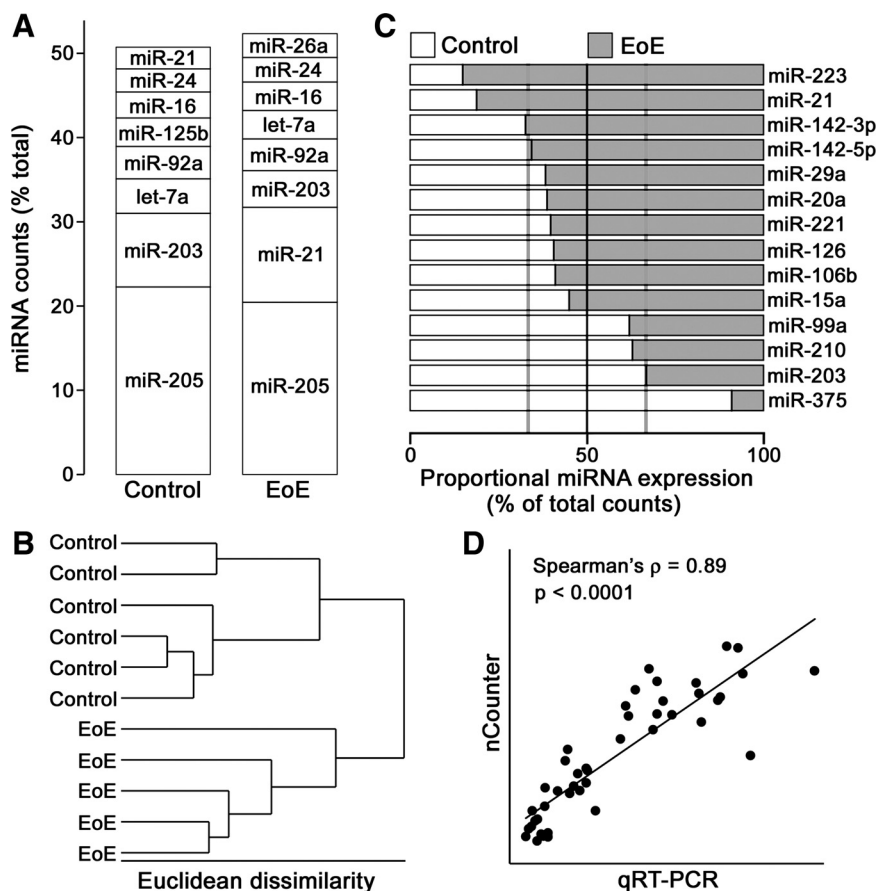
RESULTS

Profiling of esophageal miRNA in pediatric EoE. Esophageal miRNAs in biopsies obtained from pediatric patients with EoE (*n* = 5) and controls (*n* = 6) were profiled using the nCounter human version 1 miRNA expression assay kit. This platform can detect >600 human miRNAs without the need for input amplification. miR-205 was the most abundant miRNA in both sample groups, accounting for >20% of the total miRNA in the esophageal mucosa (Fig. 1A, Table 4). In both groups, the eight most-abundant miRNAs together accounted for >50% of the total miRNA detected. Hierarchical cluster analysis revealed that esophageal miRNA levels distinguish healthy esophageal tissue from esophageal tissue of EoE patients (Fig. 1B). SAM determined that 14 miRNAs were significantly altered between groups (Fig. 1C). Four miRNAs, including miR-203, the second-most-abundant miRNA in control samples, were decreased in EoE patients. To confirm the results of the profiling experiment, four differentially ex-

Table 3. Oligonucleotides used in subcloning experiments

Oligonucleotide/Primer	Sequence (5' to 3')
hU6TuD	
Top	TATGCTTACCGTAACCTGAAAGTATTTTCGATTTCTTGGCTTTATATATCTTGTGGAAGGACGAAACACCGACGGCGCTAGGATCATCT
Bottom	GCTGCAGGTAAGCTTGAGGTATATAGAAATTCACCTGCTGTATGATCCTAGCGCGCTCTTTTAT
Bottom	CTAGATAAAAAAGACGGCGCTAGGATCATAGCAGCAGGTTGGAATTCTATATACCTCAAGCTTACCTGCAGCAGATGATCCTAGCGCC
Bottom	GTGGGTGTTTCGTCCTTTCCACAAGATATATAAAGCCAAGAAATCGAAATACTTTCAAGTTACGGTAAGCA
TuDctrl	
Top	CATCAACGTGTAACACGTCATCTTATACGCCACAAAGTATTCTGGTCACAGAATACAACGTGTAACACGTCATCTTATACGCCACAAAG
Bottom	TCATCTTGTGGCGGTATAAGATGACGTGTTACAGTTGATTCTGTGACCAGAATACTTGTGGCGGTATAAGATGACGTGTTACAGTT
TuD203	
Top	CATCAACCTAGTGGTCCTCTACAAACATTTACCAAGTATTCTGGTCACAGAATACAACCTAGTGGTCCTCTACAAACATTTACCAAG
Bottom	TCATCTTGGTGAAATGTTTGTAGAGGACCACTAGGTTGATTCTGTGACCAGAATACTTGGTGAAATGTTTGTAGAGGACCACTAGGTT
203 sensor	
Top	TCGAGATAACTAGTGGTCCCAACATTTACACACACTAGTGGTCCCAACATTTCACTTATGC
Bottom	GGCGGCATAAGTGAATGTTGGGACCACTAGTGTGTGAAATGTTGGGACCACTAGTTATC
IL-15 PCR	
Forward	AATATTCTCGAGTGTAACAGAATCTGGATGCAA
Reverse	AATATTGGGCGCGCTTCTCTGTTTGTATGCTT

Fig. 1. Esophageal microRNA (miRNA) profiling in pediatric eosinophilic esophagitis (EoE). **A:** column graph displaying the 8 most-abundant miRNAs in esophageal biopsies from control and EoE patients as determined by the nCounter platform. miRNA levels are presented as percentage of total miRNA counts. **B:** dendrogram showing results of complete linkage hierarchical cluster analysis using the 74 miRNAs detected above background in each sample. **C:** column graph displaying relative levels of each of 14 miRNAs significantly altered between control and EoE samples as determined by Statistical Analysis of Microarrays. Gray vertical lines represent thresholds for a 2-fold difference in expression between groups. **D:** scatterplot of relative miR-21, miR-142-3p, miR-375, and miR-203 levels as determined by the nCounter platform and TaqMan quantitative RT-PCR (qRT-PCR) assays. Levels of the 4 miRNAs were significantly correlated between detection methods. ρ , Spearman's rank correlation coefficient.



pressed miRNAs (miR-21, miR-142-3p, miR-375, and miR-203) were measured by qRT-PCR in the same sample set. These miRNAs were selected on the basis of highest abundance among altered miRNAs in control or active EoE samples (Table 4). As shown in Fig. 1D, miRNA levels were highly correlated between platforms.

Validation of EoE-specific changes in esophageal miRNA. To confirm EoE-associated changes in esophageal miRNAs, qRT-PCR was performed on samples from a distinct set of control ($n = 22$), active EoE ($n = 22$), inactive EoE ($n = 22$), and GERD ($n = 6$) patients. A panel of five miRNAs from those identified as significantly altered in active EoE by the profiling experiment were selected for validation based on a twofold change in mean expression between controls and EoE samples. Each panel miRNA was significantly altered among the four patient groups of the validation set (Fig. 2, A and B). All panel miRNAs were altered in active EoE patients compared with inactive EoE patients and healthy controls. All three miRNAs elevated in active EoE patients were positively correlated with each other (Fig. 2C). In addition, eosinophil counts were significantly negatively correlated with miR-375 and positively correlated with miR-223 and miR-21. However, when the analysis was restricted to active EoE samples, eosinophil counts were not significantly correlated with any panel miRNA (data not shown). This finding may be due to the discontinuous nature of inflammation observed in the disease.

Esophageal miRNA levels are unchanged following treatment. Changes in esophageal miRNAs in response to corticosteroid treatment have been demonstrated in EoE patients (24).

Here, a longitudinal study of esophageal miRNA levels was performed in patients that responded ($n = 12$) or did not respond ($n = 12$) to therapeutic intervention (Table 2). All patients had a maximum eosinophil count of ≥ 15 per hpf at the initial time point. After treatment, patients were classified as responding to treatment if eosinophil counts were ≤ 10 per hpf or as nonresponding if eosinophil counts were ≥ 15 per hpf. miR-375 and miR-21 were selected for analysis, because the P values for these miRNAs were the lowest among those decreased and increased, respectively, in the case-control study. Despite a significant drop in eosinophil counts at the follow-up time point, esophageal miR-375 and miR-21 levels were not altered in the responding group (Fig. 3A). The nonresponding group had significantly higher eosinophil counts at the second time point but also showed no change in miRNA levels (Fig. 3B). When the analyses were restricted to those patients receiving only steroid treatment, miRNA levels remained unchanged.

IL-15 is not a direct target of miR-203. The reduction of miR-203 in EoE represents a dramatic shift in esophageal miRNA content, and the accompanying loss of target regulation may affect relevant pathological signaling pathways. IL-15, a cytokine required for allergen-induced inflammation, has been implicated in EoE pathogenesis (6, 33, 34, 49). Bioinformatic methods predict a miR-203 binding site in the human IL-15 3'-UTR (10, 18, 35). To determine if IL-15 mRNA is targeted by miR-203, human MCF-7 cells were transfected with a luciferase reporter construct containing a 492-bp region of the 3'-UTR of human IL-15 that includes the predicted

Table 4. *miRNA abundance in esophageal biopsies*

Healthy Controls		Active EoE Patients	
miRNA	Abundance, % total counts	miRNA	Abundance, % total counts
miR-205	23.40	miR-205	20.93
miR-203	9.15	miR-21	10.42
let-7a	4.30	miR-203	4.82
miR-92a	4.06	miR-92a	3.87
miR-125b	3.55	let-7a	3.65
miR-16	3.21	miR-16	3.40
miR-24	2.87	miR-26a	2.92
miR-21	2.73	miR-24	2.92
miR-1260	2.63	miR-1260	2.82
miR-26a	2.44	miR-125b	2.63
let-7 g	2.32	let-7 g	2.24
miR-200c	2.21	miR-142-3p	2.11
miR-23a	2.09	miR-451	2.05
miR-451	1.79	miR-23a	1.89
miR-200b	1.57	miR-126	1.84
let-7b	1.44	miR-200c	1.78
miR-375	1.43	miR-29a	1.74
miR-126	1.33	miR20a + 20b	1.60
miR-23b	1.28	let-7b	1.34
miR-99a	1.18	miR-221	1.20
miR-29a	1.14	miR-200b	1.20
miR-15b	1.11	miR-223	1.18
let-7f	1.11	let-7f	1.12
miR-20a + 20b	1.05	miR-23b	1.10
miR-30b	1.04	miR-15a	1.08
miR-142-3p	1.03	miR-30b	1.03
miR-103	1.01		

miRNA, microRNA.

miR-203 binding site. MCF-7 cells, instead of a human esophageal epithelial cell line, were selected, because they are highly amenable to efficient transfection and express elevated levels of miR-203 (21). A construct expressing a TuD RNA targeting mature miR-203 (TuD203) was used to inhibit miR-203 activity in MCF-7 cells. To demonstrate the efficacy of TuD203, MCF-7 cells were cotransfected with pMiRcheck2 expressing *Renilla* luciferase with two tandem miR-203 recognition sites in the 3'-UTR (203 sensor) and TuD203. *Renilla* luciferase expression was significantly derepressed in the presence of TuD203 (Fig. 4). However, miR-203 inhibition by TuD203 did not affect luciferase activity in cells transfected with the IL-15 3'-UTR reporter plasmid, suggesting that human IL-15 is not regulated by miR-203.

Esophageal miRNA changes are not reflected in the circulating RNA pool. Altered levels of circulating miRNAs have been reported in pediatric EoE (26). Here, circulating levels of EoE-associated esophageal miRNAs were measured in sera of control ($n = 11$), active EoE ($n = 12$), inactive EoE ($n = 12$), and GERD ($n = 12$) patients via qRT-PCR. No significant difference between groups was observed for any of the panel miRNAs (Fig. 5). Among the 27 patients for whom both esophageal and serum miRNAs were measured, there was no significant correlation between esophageal and serum levels for any of the five panel miRNAs (Fig. 6, A–C; data not shown). To determine if other circulating miRNAs are altered in pediatric EoE, serum miRNAs were profiled in patients with active EoE ($n = 5$) and inactive EoE ($n = 5$) using a low-density qRT-PCR array platform. No discernable separation between sample groups was evident following principal component analysis (Fig. 6D). SAM analysis revealed that no miRNAs

were significantly altered between sample groups (data not shown).

DISCUSSION

The present study has characterized miRNA abundance in esophageal biopsies of control and pediatric patients with EoE, an important advance in our knowledge of miRNA in this disease. In EoE patients, miR-375 showed the largest reduction

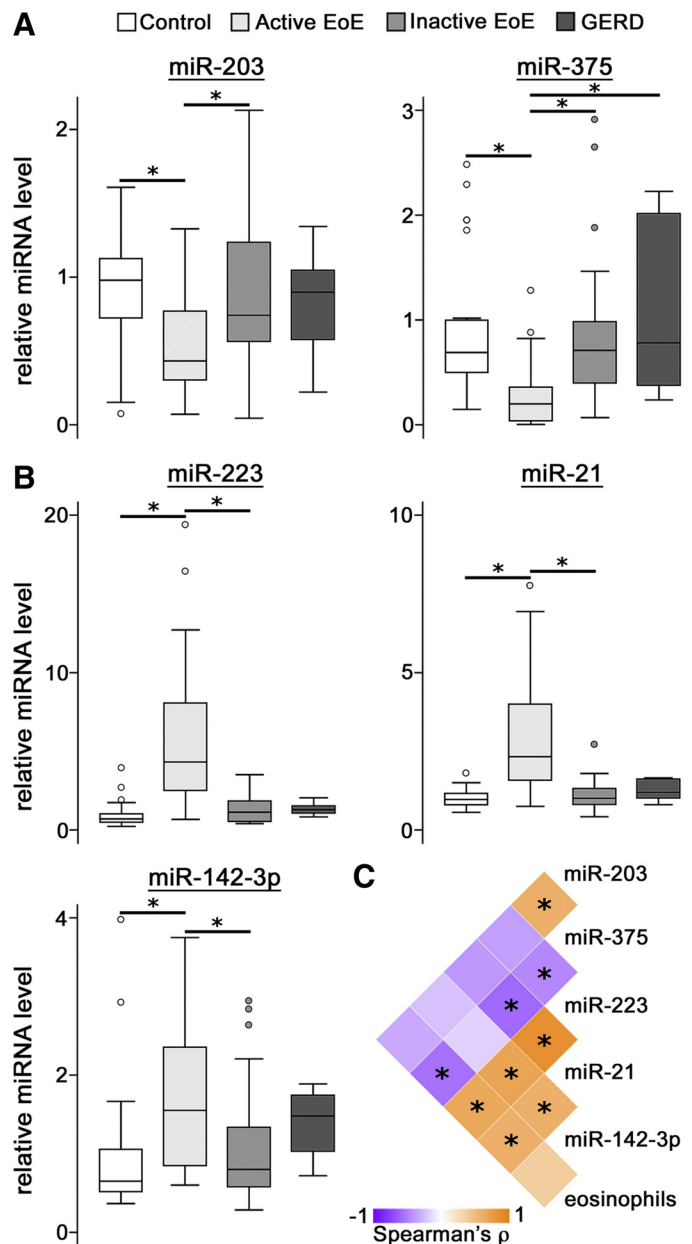
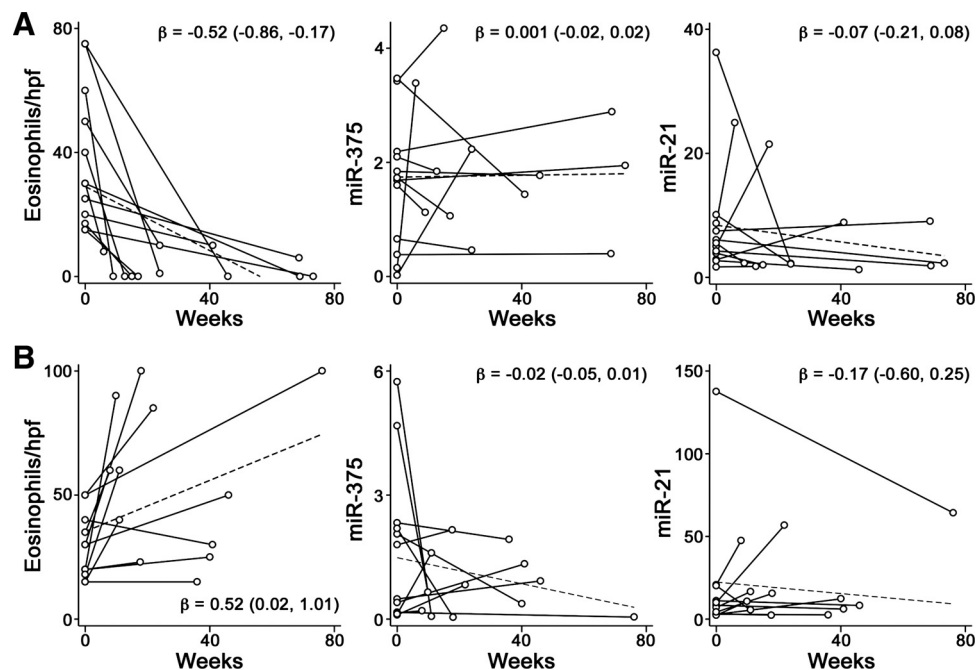


Fig. 2. Confirmation of EoE-associated esophageal miRNAs. A and B: box plots showing decreased (A) or elevated (B) relative levels of panel miRNAs in active EoE patients. miRNA levels are scaled such that the mean of control samples is equal to 1. Each miRNA was significantly altered at Kruskal-Wallis $P < 0.05$. Box, 1st–3rd quartiles; line, median; whiskers, adjacent values; ○, data points outside adjacent values. *Holm-Bonferroni-corrected $P < 0.05$. C: heat map showing correlation among panel miRNAs and eosinophil counts. Correlation among all samples was determined using the Spearman rank-order correlation test. *Bonferroni-corrected $P < 0.05$. GERD, gastroesophageal reflux disease; ρ , Spearman's rank correlation coefficient.

Fig. 3. Longitudinal study of esophageal miRNAs in EoE patients. A and B: spaghetti plots showing eosinophil and miRNA levels in esophageal biopsies from patients with improved (A) or unimproved (B) eosinophilia ($n = 12$ each) at a follow-up clinical visit. Eosinophil numbers are presented as highest count in a high-power field (hpf). miRNA levels were normalized as described in MATERIALS AND METHODS. Dashed lines indicate least-squares lines. β , Slope of the least-squares line. 95% confidence intervals of β are shown in parentheses.

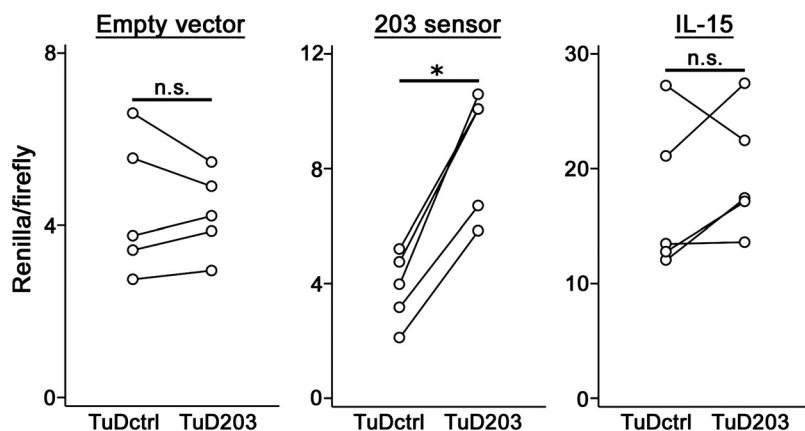


by fold change, consistent with a previous study (26). However, because miR-375 was the 17th-most-abundant miRNA in the esophageal mucosa of controls (accounting for 1.43% of measured transcripts), the more abundant miR-21 and miR-203 showed larger changes in transcript numbers. The second-most-abundant esophageal miRNA in control subjects, miR-203, was reduced twofold in EoE. This reduction of miR-203 may reflect the basal layer hyperplasia and the loss of differentiated epithelium observed in EoE. Indeed, miR-203 expression is over fivefold higher in the differentiated epithelium than the basal layer in the human esophagus (56). However, basal layer hyperplasia is also a prominent feature of reflux esophagitis, and no difference in miR-203 was observed between control and GERD patients. In contrast, the large increase in miR-21, miR-223, and miR-142-3p transcripts in the EoE mucosa may reflect the influx and expansion of immune cells characteristic of the disease, as these miRNAs are highly expressed in myeloid and lymphoid cell populations (8, 25, 49). miR-21 is widely expressed and is abundant in differentiated epithelial cells and basal cells of the human esophagus (56); hence, the increase in EoE tissue may, instead, result

from expansion of the basal layer. Transcripts targeted by miR-21, including transforming growth factor- β 1 and suppressor of cytokine signaling 5, may play an important role in pediatric EoE pathogenesis (12, 14, 27).

Esophageal miRNA-203 expression is elevated in the differentiated epithelium layer compared with underlying basal cells (56). The reduction of miR-203 in EoE may alter immune cell regulation. miR-203 has been shown to repress the expression of inflammatory modulators, including TNF α , IL-24, and suppressor of cytokine signaling 3, in keratinocytes (36). IL-15, a γ -chain family cytokine produced by several cell types, including keratinocytes, is required for allergen-induced T helper type 2 (Th2) cell immune responses *in vivo* (39, 40). IL-15 mRNA levels are elevated in the esophagus of pediatric EoE patients, and IL-15 induces epithelial eotaxin production in a mouse model of EoE (7, 57). The 3'-UTR of IL-15 contains a predicted miR-203 binding site, but miR-203 was not found to negatively regulate IL-15 *in vitro*. Although no evidence of transcript regulation was identified via luciferase assay, definitive evidence will require experiments detecting endogenous mRNA and protein levels following miR-203

Fig. 4. Effect of miR-203 on the IL-15 3'-untranslated region (UTR). Paired dot plots show *Renilla*/firefly luciferase activity in MCF-7 human breast adenocarcinoma epithelial cells cotransfected with a luciferase reporter and a target decoy (TuD) construct. TuD203 expression construct significantly increased *Renilla* luciferase activity in cells cotransfected with the 203 sensor construct, but not in cells cotransfected with an empty vector or the IL-15 construct. Each data point represents mean of 3 technical replicates. Lines indicate paired experiments performed in parallel. * $P < 0.05$ (Wilcoxon's signed-rank test). ns, No significant difference between groups. TuDctrl, TuD containing scrambled sequence binding sites; TuD203, TuD containing miR-203 binding sites.



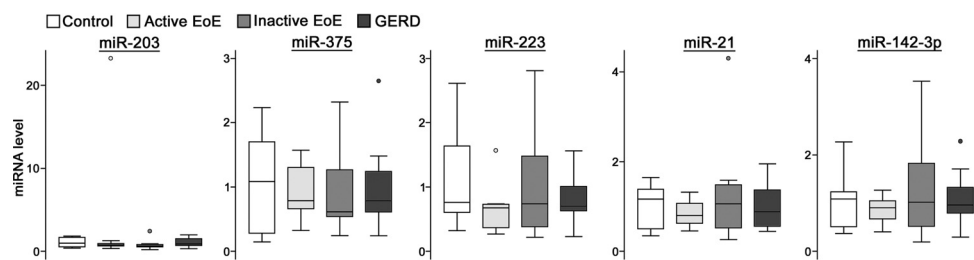


Fig. 5. Serum miRNAs in pediatric EoE. Box plots display relative serum levels of the 5 panel miRNAs. miRNA levels were normalized as described in MATERIALS AND METHODS and scaled such that the mean of control samples is equal to 1. No significant differences in miRNA levels were observed among the sample groups. Box, 1st–3rd quartiles; line, median; whiskers, adjacent values; ○, data points outside adjacent values.

perturbation in human esophageal epithelial cells. Interestingly, a single-nucleotide polymorphism (rs10519613) located in the predicted miR-203 binding site of the IL-15 3'-UTR is associated with an increased risk of psoriasis vulgaris (55), pediatric asthma (4), and minimal residual disease (52). The possibility remains that rs10519613 alters miR-203-loaded RNA-induced silencing complex functionality at this location. Recent studies have demonstrated that overexpression of miR-203 alters the expression of IL-6, a Th2 cytokine elevated in the plasma of EoE patients (6, 42, 48). Studies are needed to characterize the miR-203 target transcriptome and ascertain the pathophysiological consequences of reduced miR-203 in pediatric EoE. In addition, studies examining the modulation of esophageal miRNA expression by cytokines linked to EoE pathogenesis, including the Th2 cytokines IL-15 and IL-13, may provide valuable insight (20, 29).

Several miRNAs identified here as significantly altered in pediatric EoE have been associated with EoE. Of the 32 miRNAs found by T. X. Lu et al. (26) to be altered in EoE esophageal biopsies, 7 are among the 14 miRNAs identified by the profiling experiment performed here (Table 5). In addition, T. X. Lu et al. identified miR-106b*, the passenger strand miRNA of miR-106b, as upregulated in EoE. Subsequently, S. Lu et al. (24) measured 12 miRNAs, including miR-142-3p, miR-21, and miR-203, in esophageal biopsies obtained from pediatric EoE patients and healthy controls. miR-21 was increased and miR-203 was decreased in EoE, in accordance with the results presented here and by T. X. Lu et al. In contrast, S. Lu et al. found no difference in miR-142-3p levels between groups.

T. X. Lu et al. (26) reported that 27 of 32 esophageal miRNAs altered in active EoE were unaffected in patients with inactive disease. Similarly, all five of the miRNAs chosen for validation in the present case-control study were significantly altered between active and inactive EoE patients. In a longitudinal study, S. Lu et al. (24) profiled miRNAs in esophageal biopsies in EoE patients before and after corticosteroid treatment and found that 32 miRNAs were upregulated and 4 were downregulated following treatment (Table 5). However, no significant change in esophageal miR-375 or miR-21 was observed in the present longitudinal study of responding or nonresponding patients. This finding seemingly contradicts the longitudinal study by S. Lu et al. and the differential expression observed in the present case-control study and the study of T. X. Lu et al. The possibility remains that therapeutic intervention more rapidly alters intraepithelial eosinophil numbers than miRNA levels. A more thorough examination of temporal changes in esophageal miRNAs during treatment is necessary to address this issue.

Three miRNAs, miR-375, miR-21, and miR-223, were found to significantly correlate with peak eosinophils per hpf,

consistent with the findings of T. X. Lu et al. (26). However, in contrast to the study of T. X. Lu et al., miR-203 and miR-142-3p levels in the present study did not correlate with eosinophils per hpf. The relationship between tissue miRNA and eosinophil counts presents the possibility for a novel diagnostic and surrogate biomarker, as tissue miRNA expression changes or miRNA release following injury can be reflected in the circulating miRNA pool (22, 32, 46). This corollary offers the possibility of a novel diagnostic and surrogate biomarker for EoE, for which invasive endoscopic testing is currently used for diagnosis and management. Indeed, T. X. Lu et al. identified an increase in miR-146a, miR-146b, and miR-223 in the plasma of EoE patients compared with healthy controls (26). In contrast, in the present experiments, no significant difference in serum miRNAs between the four sample groups was found. Although different starting materials and normalization techniques were used, these factors alone are unlikely to explain these contradictory

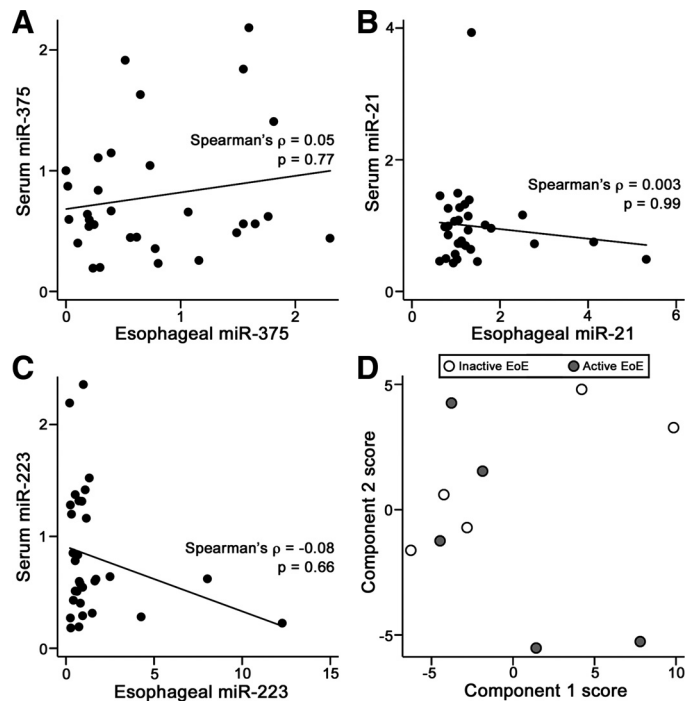


Fig. 6. Serum miRNAs in pediatric EoE. A–C: scatterplots of miR-375, miR-21, and miR-223 levels in serum and esophagus of 31 patients. Each data point indicates a single subject. Solid lines indicate linear least-squares lines. miRNA levels were scaled such that the mean of control samples is equal to 1. D: score plot showing the first 2 components of a principal component analysis using data from the 80 serum miRNAs detected in all active and inactive EoE samples ($n = 5$ each). Components 1 and 2 together account for >52% of total variance. ρ , Spearman's rank correlation coefficient. No discernable separation is observed between active and inactive EoE samples.

Table 5. Summary of esophageal miRNA expression in EoE

	Previous Studies		Present Study
	T. X. Lu et al. (26)	S. Lu et al. (24)	
Array comparison	aEoE (<i>n</i> = 10), HC (<i>n</i> = 8), iEoE (<i>n</i> = 6), CE (<i>n</i> = 5)	aEoE (<i>n</i> = 7), iEoE (<i>n</i> = 7) (longitudinal study)	aEoE (<i>n</i> = 5), HC (<i>n</i> = 6)
Platform	Life Technologies TaqMan array	Life Technologies TaqMan array	nanoString nCounter
aEoE vs. HC	Upregulated: miR-886-5p, miR-886-3p, miR-222*, miR-7, miR-29b, miR-642, miR-339-5p, miR-21, miR-21*, miR-142-5p, miR-146a, miR-146b, miR-142-3p, miR-132, miR-592, miR-92a-1*, miR-223*, miR-223, miR-801, miR-106b* Downregulated: miR-375, miR-211, miR-210, miR-365, miR-203, miR-193a-5p, miR-193b, miR-193a-3p, let-7c, miR-144*, miR-30a-3p	N/A	Upregulated: miR-223, miR-21, miR-142-3p, miR-142-5p, miR-29a, miR-20a, miR-221, miR-126, miR-106b, miR-15a Downregulated: miR-99a, miR-210, miR-203, miR-375
aEoE vs. iEoE	Upregulated: miR-886-5p, miR-886-3p, miR-222*, miR-21, miR-21*, miR-142-5p, miR-146a, miR-146b, miR-142-3p, miR-132, miR-592, miR-223*, miR-223, miR-801, miR-106b* Downregulated: miR-375, miR-211, miR-210, miR-365, miR-203, miR-193a-5p, miR-193b, miR-193a-3p, let-7c, miR-144*, miR-30a-3p	Upregulated: miR-214, miR-146b-5p, miR-146a, miR-145, miR-142-3p, miR-21, miR-29b, miR-339-3p, miR-100, miR-10a, miR-223, miR-324-5p, miR-99a, miR-34a, miR-195, miR-125-5p, miR-140-5p, miR-502-3p, miR-150, miR-29a, miR-99b, miR-139-3p, miR-126, miR-342-3p, miR-181a, miR-93, miR-27a, miR-152, miR-27b, miR-152, miR-27b, miR-29c, miR-222, miR-155 Downregulated: miR-361-5p, miR-147, miR-203, miR-489	N/A
Validation comparison	aEoE (<i>n</i> = 7–17), HC (<i>n</i> = 7–17)	aEoE (<i>n</i> = 8), HC (<i>n</i> = 10)	aEoE (<i>n</i> = 22), HC (<i>n</i> = 22), iEoE (<i>n</i> = 22), GERD (<i>n</i> = 6)
Platform	Life Technologies TaqMan RT-PCR	Life Technologies TaqMan RT-PCR	Life Technologies TaqMan RT-PCR
aEoE vs. HC	Upregulated: miR-21, miR-223 Downregulated: miR-375, let-7c, miR-203	Upregulated: miR-214, miR-146b-5p, miR-146a, miR-21 Downregulated: miR-203, miR-489	Upregulated: miR-223, miR-21, miR-142-3p Downregulated: miR-203, miR-375
Age of aEoE subjects, yr	9.3 (1.9–32.3)	7.8 (1.3–16.0)	10.8 (1.7–18.0)

Age is presented as mean (range). aEoE, active EoE; HC, healthy controls; iEoE, inactive EoE; CE, chronic esophagitis; N/A, not applicable.

findings. The need for reliable, noninvasive biomarkers of EoE warrants continued investigation of circulating miRNA in this patient population.

Several limitations to the current study should be considered. Because of the discontinuous nature of inflammation in EoE, disease activity may differ between biopsies used for histological observation and those processed for RNA analyses. This may in part explain the discrepancies between the present study and previous studies. In addition, sample sizes in the present study precluded subgroup analyses for lack of statistical power. miRNA changes identified in pediatric patients may not occur in the adult population; no reports have addressed human miRNAs in adult EoE patients. Finally, newly discovered human miRNAs are not measured by the nCounter human version 1 platform; sequencing of esophageal miRNAs in EoE patients may identify additional disease-specific changes. Recent increases in EoE diagnoses in children warrant continued investigation of the role of miRNAs in the disease.

ACKNOWLEDGMENTS

The authors thank the patients who generously gave their consent to participate in the study.

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GRANTS

This study was funded in part by the American Partnership For Eosinophilic Disorders. A. M. Zahm is supported by National Institute of Diabetes and Digestive and Kidney Diseases Ruth L. Kirschstein National Research Service Award for Individual Postdoctoral Fellows (F32).

DISCLOSURES

J. R. Friedman is currently employed at Janssen Research and Development.

AUTHOR CONTRIBUTIONS

A.M.Z., C.M.-K., and J.R.F. are responsible for conception and design of the research; A.J.B. recruited the study subjects and managed specimen and clinical data collection and storage; A.M.Z., C.M.-K., D.M.T., C.L.L.G., and N.J.H. performed the experiments; A.M.Z., C.M.-K., and C.L.L.G. analyzed the data; A.M.Z., C.M.-K., D.M.T., C.L.L.G., and J.R.F. interpreted the results of the experiments; A.M.Z. prepared the figures; A.M.Z. and C.M.-K. drafted the manuscript; A.M.Z., C.M.-K., D.M.T., C.L.L.G., N.J.H., and J.R.F. approved the final version of the manuscript; C.M.-K., N.J.H., and J.R.F. edited and revised the manuscript.

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