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Proton-pump Inhibitor Response Prediction Using Esophageal microRNAs in Children With Eosinophilic Esophagitis

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ABSTRACT

Objectives: Eosinophilic esophagitis (EoE) is a chronic esophageal disease characterized by eosinophilic inflammation. Proton-pump inhibitors (PPI) induce disease remission but no predictive factors of PPI-responsiveness have been identified yet. So, a biomarker must be found to differentiate between responders (PPI-R) and nonresponder patients (PPI-NR) to PPI. Aims were to identify any molecular biomarker that could predict PPI responsiveness and to study molecular remission after PPI therapy.

Methods: This prospective study enrolled 39 controls and 43 pediatric children with EoE from 2 hospitals, and they were treated with esomeprazole for 8 to 12 weeks. After therapy, patients were classified as either PPI-R or PPI-NR. Biopsies were collected and RNA, microRNAs, and proteins were isolated from them, measuring levels by qPCR and Western blot (WB). Also, miRNAs were evaluated in serum.

Results: We found several esophageal miRNAs with different expression values between PPI-R and PPI-NR children, which can be used to discriminate them (area under curve = 0.90). No useful serum miRNAs were, however, identified. Also, these miRNAs were dysregulated in responder patients before and after PPI therapy. Moreover, we corroborated in this child population, that PPI-R displayed a significant decrease in eotaxin-3, IL-5, IL-13, periostin, and major basic protein ($P < 0.05$) and a significant increase in filaggrin levels after PPI treatment ($P < 0.01$).

Conclusions: Esophageal miRNA levels found are able to discriminate between both PPI-R and PPI-NR at baseline, and before and after treatment in PPI-R, so they could be used as biomarkers. Furthermore, we observed clinical and esophageal molecular restoration in PPI-R patients after PPI therapy.

Key Words: eosinophilic esophagitis, esophagus, microRNA, proton-pump inhibitor, responsiveness

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What Is Known

- Proton-pump inhibitors are accepted as a first-line therapy to treat eosinophilic esophagitis.
- Proton-pump inhibitors produce molecular changes in patients with eosinophilic esophagitis.
- A good biomarker to identify proton-pump inhibitor-response is needed to monitor the treatment and differentiate responders from nonresponders.

What Is New

- This study shows a microRNA profile that is able to differentiate responders from nonresponder patients treated with esomeprazole.
- Treatment produces an esophageal microRNA alteration in responder patients, showing a microRNA restoration after therapy administration.
- Further investigation is needed to assay if this miRNA profile may translate to clinical treatment monitoring of pediatric patients with eosinophilic esophagitis.

Eosinophilic esophagitis (EoE) is a chronic immune-mediated esophageal disease characterized clinically by symptoms of esophageal dysfunction and histologically by eosinophil-predominant inflammation restricted to the esophagus, with biopsies of the

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esophageal mucosa showing 15 or more eosinophils per high-power field (eos/hpf) (1).

Three first-line therapies are used: proton-pump inhibitors (PPI), swallowed topical corticosteroids, and elimination diets (2). Recently, several guidelines advise to use PPI therapy for EoE treatment, based on different observational reports that show a decrease in histologic findings linked to disease from 42% of patients (3). The moderate efficacy, low cost, ease of administration, and safety profile of PPI make it the treatment of choice (4).

Current recommendations for EoE diagnosis and monitoring require repeated endoscopic examinations with biopsies; therefore, less invasive methods to diagnose and supervise treatment response are desirable; but to date, no predictive factors of response to PPIs have been identified. Several molecules involved in EoE pathophysiology, such as interleukin (IL)-5, eotaxin-3, and filaggrin have been suggested as biomarkers (5,6); however, to date no optimal biomarker is available yet. In this sense, microRNAs (miRNAs) are molecules that could be good biomarkers in EoE. They are small noncoding RNA molecules and 18 to 22 nucleotides in length, acting as regulators of gene expression (7). MiRNAs are associated with several diseases and they are considered excellent biomarkers and diagnostic tools for many diseases (8), because of optimal features: abundance, stability, ubiquity, easy to detect, their association to a pathological status and they could be routinely used in clinical practice (9). For these reasons, we hypothesized that some miRNAs could be identified that predict PPI responsiveness.

Therefore, the main objective of our study is to examine the molecular differences between children with different response to PPI therapy in order to establish molecular biomarkers that can be used to distinguish responders (PPI-R) from nonresponder (PPI-NR) patients, avoiding the selection of an ineffective treatment.

METHODS

Please refer to Supplemental Digital Content 1, *http://links.lww.com/MPG/B984* for a complete detailed description of the study methods used.

Subjects and Study Design

Forty-three children from 3 to 15 years of age who were diagnosed with EoE (10), were recruited from the pediatric gastroenterology unit of 2 hospitals in Madrid: Hospital Universitario Puerta de Hierro-Majadahonda and Hospital Universitario Severo Ochoa. From the whole EoE population, esomeprazole was administered to 27 of these patients at 1 mg/kg per dose twice daily, with a maximum dose of 40 mg given twice a day, throughout 8 to 12 weeks (10), and the rest was treated with elimination diet or topical swallowed corticosteroids. Samples from these patients medicated with PPI were used to identify potential candidate genes, proteins, and microRNAs. We included as controls 39 pediatric subjects without previous EoE diagnosis.

This prospective study was conducted in accordance with the principles set forth in the Declaration of Helsinki and was approved by the ethics committee at the Hospital Universitario Puerta de Hierro-Majadahonda and Hospital Universitario Severo Ochoa. All subjects were properly advised in writing and provided signed informed consent.

Endoscopy and Histology

An upper gastrointestinal endoscopy was performed at baseline and after 8 to 12 weeks of PPI monotherapy in EoE patients. Esophageal eosinophils were counted in a single high-power field (hpf) of 400× magnification corresponding to an area of 0.24 mm^2. Esophageal eosinophilia was defined as the presence of ≥15 eos/hpf in 1 or more biopsy samples. Histological remission was defined as less than 15 eos/hpf in all the examined biopsies.

Serum Samples

Serum was obtained by blood clotting and centrifugation at 3000 rpm for 10 min at 4 °C and stored at −80 °C until use.

RNA, MicroRNAs, and Protein Isolation

Esophageal biopsies were homogenized and total tissue RNA (including miRNAs) and proteins were purified from tissue samples, as previously described (11). Also, miRNAs from serum were obtained using miRNeasy serum/plasma advanced kit (Qiagen, Hilden, Germany).

Next Generation Sequencing

RNA was extracted from 5 esophageal biopsies obtained from PPI-R subjects before and after treatment. RNA was used to perform miR-seq, according to NGS protocol (12).

Reverse Transcription PCR, TaqMan Gene Expression Assays, and miRNA Validation

RNA was quantified in a Nanodrop ND-1000 (Bonsai Advanced, Madrid, Spain). Then, 1 μg of RNA was reverse-transcribed into cDNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher, Waltham, MA), and semi-quantitative real-time PCR (qRT-PCR) was carried out using specific TaqMan gene expression assay probes (Qiagen).

MiRNAs were retrotranscribed to cDNA by miRCURY LNA RT Kit (Qiagen), and they were evaluated by RT-qPCR using miRCURY LNA SYBR Green PCR Kit (Qiagen), using suitable probes (Supplemental Digital Content 2, *http://links.lww.com/MPG/B985*).

All samples were run in triplicate, and genes and miRNAs were analyzed using Ct value and relative expression was calculated by means of the $2^{-\Delta\Delta Ct}$ method (13).

Western Blot Analysis

Isolated proteins from esophageal samples were quantified according to the bicinchoninic acid (BCA) method (14). Twenty micrograms of total protein per lane was resolved on SDS-PAGE and analyzed by Western blot (WB), and several proteins were evaluated. β-actin was used as protein-loading control.

Bioinformatic Analyses

Multivariate analyses of differentially expressed genes and miRNAs were carried out with ClustVis bioinformatic tool (15) by principal component analysis (PCA) and unsupervised hierarchical clustering heatmap analysis.

Statistical Analyses

Clinical characteristics are expressed as mean, standard deviation (SD), median, and range, and comparisons were performed with Fisher exact test. Results are expressed as mean ± SD. Normality was analyzed using the Shapiro-Wilk test. Parametric data comparison between nonpaired groups was performed using

unpaired *t* test. Nonparametric and nonpaired groups were compared by using Mann-Whitney test. Comparison between paired groups was made with paired *t* test for parametric data and with Wilcoxon matched paired test for nonparametric data.

A multivariate logistic regression model was obtained using previous miRNA univariate logistic regression models with a $P < 0.25$. $P < 0.05$ was considered significant.

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA). A multivariate logistic regression model was performed using R (*https://www.r-project.org/*).

RESULTS

Proton-pump Inhibitor Treatment Improves Clinical Symptoms and Endoscopic and Histological Findings

A total of 39 children with no EoE diagnosis and 43 pediatric patients diagnosed with EoE were included in the study. Supplemental Digital Content 3 summarizes the demographic and clinical characteristics of controls and patients with EoE, *http://links.lww.com/MPG/B986*.

From the whole EoE population, 27 patients were treated with PPI for 8 to 12 weeks as described previously. After PPI administration, 17 subjects (63%) achieved histological remission. A total of 10 patients were PPI nonresponders. Table 1 summarizes the demographic, clinical, endoscopic, and histologic findings in PPI-R and PPI-NR patients. Additionally, Table 1 shows the range of esomeprazole daily dose administered to the EoE children.

Differential Gene Expression Between Controls and Eosinophilic Esophagitis Patients

In order to establish the molecular characterization of patients, we selected the most relevant genes involved in EoE pathology and compared their expression between controls (n = 21) and some pediatric subjects with EoE randomly selected from the whole EoE population (n = 27). A set of 16 genes were evaluated, and statistically differential expression was found in 15 of them (Supplemental Digital Content 4A, *http://links.lww.com/*

TABLE 1. Demographic and clinical characteristics of patients with eosinophilic esophagitis grouped in proton-pump inhibitor-responder and proton-pump inhibitor-nonresponder patients before and after esomeprazole therapy

	PPI-R		PPI-NR	
	PRE (n = 17)	POST (n = 17)	PRE (n = 10)	POST (n = 10)
Male sex, no. (%)	13 (76.47)	13 (76.47)	8 (80.00)	8 (80.00)
Age (y), mean ± SD	10.73 ± 3.08	10.93 ± 3.08	10.87 ± 4.00	11.03 ± 3.82
IgE, kU/L, median (range)	132.00 (10–5000)	290.00 (16–5000)	226.50 (31–2895)	448.50 (87–3102)
Peripheral blood eosinophils, eos/mm^3, mean ± SD	0.67 ± 0.34	0.52 ± 0.53	0.64 ± 0.39	0.61 ± 0.33
Esomeprazole daily dose, mg, range	40–80	NA	40–80	NA
Atopic disease, no. (%)				
Atopy	11 (64.71)	NA	7 (70.00)	NA
Atopic dermatitis	5 (29.41)	NA	5 (50.00)	NA
Asthma	7 (41.18)	NA	4 (40.00)	NA
Food allergy	3 (17.65)	NA	5 (50.00)	NA
Allergic rhinoconjunctivitis	6 (35.29)	NA	5 (50.00)	NA
Symptoms, no (%)				
Heartburn	9 (52.94)	0 (0.00)***	4 (40.00)	3 (30.00)
Food impaction	9 (52.94)	0 (0.00)***	6 (60.00)	0 (0.00)*
Abdominal pain	6 (35.29)	1 (5.88)	4 (40.00)	1 (10.00)
Vomiting	6 (35.29)	1 (5.88)	1 (10.00)	1 (10.00)
Dysphagia	10 (58.82)	0 (0.00)***	6 (60.00)	2 (20.00)
GERD	4 (23.53)	4 (23.53)	0 (0.00)	0 (0.00)
Endoscopic signs, no. (%)				
Rings	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
White exudates	14 (82.35)	2 (11.76)****	10 (100.00)	7 (70.00)
Longitudinal furrows	17 (100)	8 (47.06)***	10 (100.00)	10 (100.00)
Narrowing	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Crêpe-paper	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Erosive esophagitis	1 (5.88)	0 (0.00)	0 (0.00)	0 (0.00)
Histologic findings				
Eosinophil counts, eos/hpf, mean ± SD				
Distal	58.29 ± 29.29	3.88 ± 4.72****	68.10 ± 35.93	57.40 ± 38.20
Upper-mid	57.71 ± 28.06	3.88 ± 4.46****	71.30 ± 33.68	36.70 ± 26.19*

Comparisons were performed between PPI-R before and after treatment, and PPI-NR before and after esomeprazole administration. Eos/hpf, eosinophils per high-power field; GERD = gastroesophageal reflux disease; IgE = immunoglobulin E; PPI-NR = proton-pump inhibitor-nonresponders; PPI-R = proton-pump inhibitor-responders; POST = after treatment; PRE = before treatment; SD = standard deviation.

*$P < 0.05$.

***$P < 0.001$.

****$P < 0.0001$.

MPG/B987), including upregulated genes of cytokines and chemokines (*CCL26*, *IL5*, *IL13, IL33,* and *TSLP*), eosinophil-protein-specific genes (*RNASE3* and *PRG2*), and other molecules associated with EoE and Th2 response (*POSTN*). Furthermore, *FLG* was downregulated, showing epithelial barrier dysfunction.

Gene and Protein Profiles Were Restored in Responders After Protein Pump Inhibitor Treatment, But Did Not Discriminate Between Protein Pump Inhibitor Responder and Protein Pump Inhibitor Nonresponder Patients at Baseline

For the purpose to examine molecular restoration in EoE subjects and to find a molecular signature, which could discriminate between responders and nonresponders, we compared the set of 15 genes in both subgroups of patients: PPI-R ($n = 12$) and PPI-NR ($n = 8$) before and after treatment. Figure 1 shows the genes altered in a significant manner.

In PPI-R patients, 5 genes were differentially downregulated after PPI therapy: *CCL26* ($P < 0.01$; Fig. 1A), *IL5* ($P < 0.05$; Fig. 1B), *IL13* ($P < 0.05$; Fig. 1C), *POSTN* ($P < 0.01$; Fig. 1D), and *PRG2* ($P < 0.05$; Fig. 1E); 1 was upregulated *FLG* ($P < 0.01$; Fig. 1F).

In order to corroborate these changes, we evaluated protein levels of IL-5, eotaxin-3, MBP, and filaggrin by WB (Supplemental Digital Content 5, *http://links.lww.com/MPG/B988*) in PPI-R subjects, showing a significant reduction in eotaxin-3 and MBP protein levels after treatment ($P < 0.05$; Supplemental Digital Content 5A–B, *http://links.lww.com/MPG/B988*) and higher levels of filaggrin after PPI-administration ($P < 0.05$; Supplemental Digital Content 5C, *http://links.lww.com/MPG/B988*). Although IL-5 protein levels tended to decrease after treatment, no significant changes were observed ($P > 0.05$; Supplemental Digital Content 5D, *http://links.lww.com/MPG/B988*).

In the PPI nonresponder population (Fig. 1), we observed a significant reduction in gene expression of 3 molecules after PPI therapy: *CCL26* ($P < 0.05$; Fig. 1A), *IL13* ($P < 0.01$; Fig. 1C), and *POSTN* ($P < 0.05$; Fig. 1D). The changes found in *IL5* (Fig. 1C), *PRG2* (Fig. 1E), and *FLG* (Fig. 1F) were not significant in this nonresponder population.

In view of these results, we decided to perform a PCA with these 6 altered genes (Fig. 1), comparing them between PPI-R and PPI-NR at baseline (Fig. 1G) and in responder patients according to PPI administration (Fig. 1H). This gene signature, however, did not differentiate between the 2 groups at baseline.

Esophageal MicroRNA Level Restoration After Proton-pump Inhibitor Treatment

Total RNA enriched in miRNA from 5 pairs of biopsies from PPI-R children before and after PPI treatment was analyzed by NGS. Among all the miRNAs checked, we found that 116 miRNAs were differentially expressed in esophageal biopsies: 6 were upregulated and 110 were downregulated after PPI treatment. These miRNAs were selected based on a *P* value of less than 0.05 and a fold change higher than 1.5. The complete list of differential miRNAs appears in Supplemental Digital Content 6, *http://links.lww.com/MPG/B989*.

To validate the results of miRNA expression, we tested them in 15 pairs of biopsies from PPI-R children before and after treatment using qRT-PCR. We analyzed 6 miRNAs upregulated in children after treatment (miR-4485-3p, miR-135a-2-3p, miR-3659, miR-135a-5p, miR-31-3p, and miR-664a-3p) and 11 downregulated miRNAs (miR-520d-5p, miR-520a-5p, miR-525-5p, miR-519d-5p, miR-4773, miR-522-3p, miR-490-3p, miR-137-3p, miR-223-3p, miR-212-5p, and miR-7-5p), which were selected based on several criteria: differential expression with a *P* value less than 0.05 and a fold change higher than 1.5; putative target genes associated with the pathology; and their implication in the disease, according to previous studies (16). In addition, we decided to check 2 additional miRNAs: miR-375-3p (Log_2 fold change = 2.17; *P* value = 0.06) and miR-21-3p (Log_2 fold change = −1.08; *P* value = 0.09). Although they did not meet the requirements of *P* value and/or fold change, we included them in the study because of their biological relevance in the disease and in inflammatory processes, as previous studies shown (17,18).

As Figure 2 shows, we confirmed that 4 miRNAs were downregulated (miR-664a-3p, $P < 0.001$; miR-7-5p, $P < 0.001$; miR-223-3p, $P < 0.0001$; and miR-21-3p, $P < 0.001$; Fig. 2A–D) and 1 miRNA was upregulated (miR-375-3p, $P < 0.01$; Fig. 2E) in PPI-R children after treatment with regard to values recorded before therapy. In PPI-NR children, we found that only 2 miRNAs were not significantly deregulated in these patients before and after treatment (miR-664a-3p and miR-223-3p, Fig. 2A and C).

Regarding the rest of the miRNAs, some were not detected by qRT-PCR, and others did not show any significant differences (miR-4485-3p, miR-135a-5p, and miR-31-3p; data not shown).

Esophageal MicroRNAs Differentiate Proton-pump Inhibitor-responder Versus Proton-pump Inhibitor-nonresponder and Controls Versus Eosinophilic Esophagitis

Analyzing these results and performing a comparison between PPI-R and PPI-NR at baseline, we observed that almost all miRNAs, with the exception of miR-21-3p, were differentially deregulated between these groups ($P < 0.05$, $P < 0.01$, Fig. 2). This fact could indicate a molecular difference between responders and nonresponders to PPI, which has not been observed to date.

To elucidate this molecular characteristic, we performed an unsupervised hierarchical clustering heatmap analysis with the 5 deregulated miRNAs. When we compared baseline miRNA levels between the group of responders and nonresponders to PPI therapy, the heatmap exhibited a good discrimination between the 2 groups (Fig. 2F). Also, the expression values of these miRNAs allowed highly precise differentiation between PPI-R patients before and after PPI treatment (Fig. 2G). Additionally, we performed an unsupervised hierarchical clustering heatmap analysis with deregulated miRNAs, showing different miRNA expression levels between controls and EoE patients (Supplemental Digital Content 4B, *http://links.lww.com/MPG/B987*).

On the basis of these findings, we created a multivariate logistic regression model to test whether the levels of these miRNAs were able to differentiate PPI-R from PPI-NR at baseline before treatment (Table 2).

After combined expression of miR-7-5p, miR-375-3p, and miR-223-3p, the resulting model improved the differentiation of PPI-R and PPI-NR patients at baseline, with an area under curve (AUC) of 0.90, demonstrating that expression values of these miRNAs can discriminate between PPI responders and non-responders at baseline.

Correlation of MicroRNA Expression Levels and Esophageal Eosinophil Counts

Interestingly, the deregulation of esophageal miRNAs between PPI-R and PPI-NR could be a fundamental difference

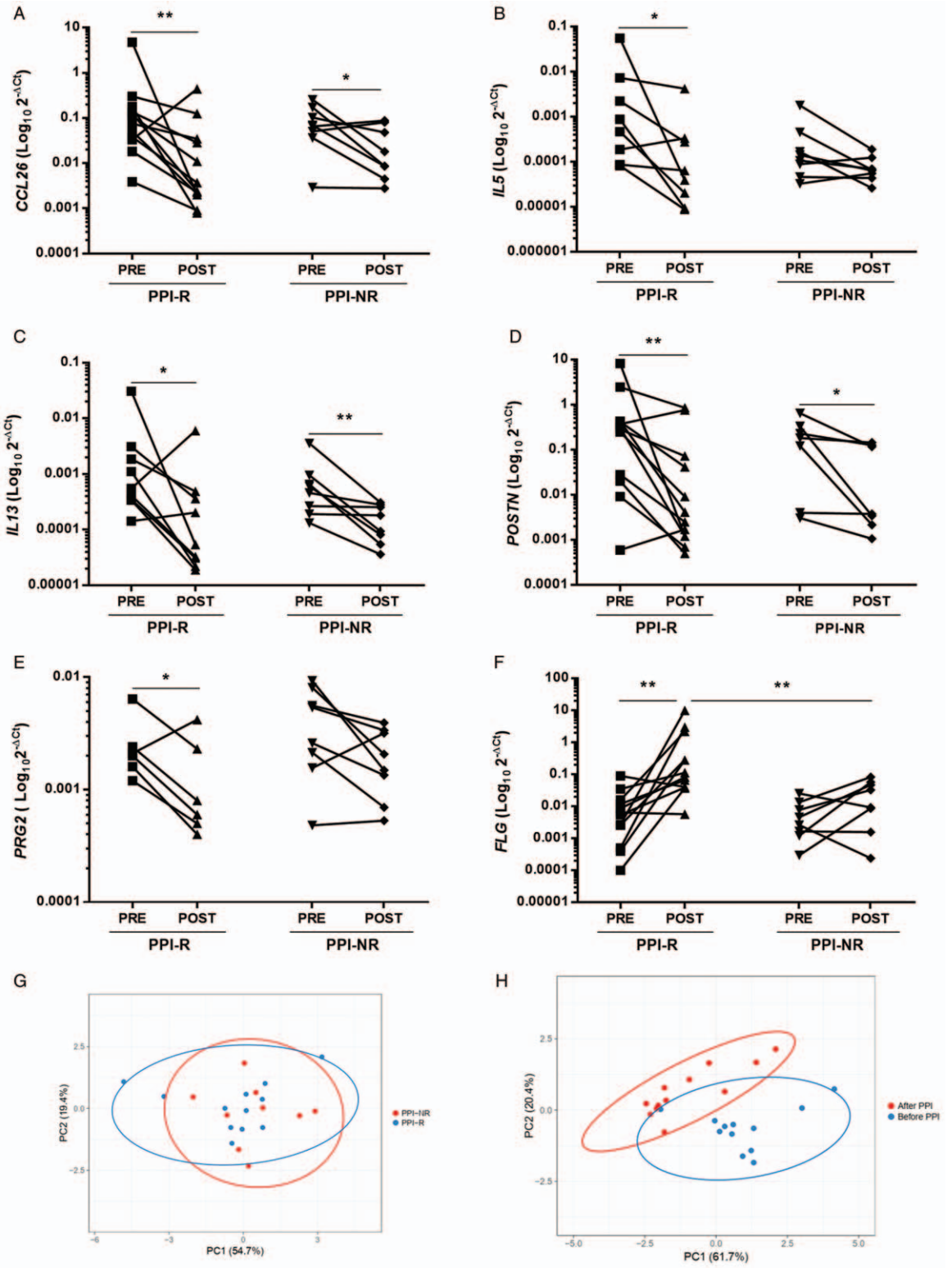

FIGURE 1. Proton-pump inhibitor treatment restores gene levels in responders but does not allow to discriminate between proton-pump inhibitor-responder and proton-pump inhibitor-nonresponder patients at baseline. Esomeprazole induces a downregulation of *CCL26, IL5, IL13, PRG2,* and *POSTN,* and an upregulation of *FLG* in PPI-R patients after PPI-administration (n = 12; A–F). *GAPDH* was used as normalization gene. All experiments were performed in triplicate. Relative gene expression is expressed as $Log_{10}\ 2^{-\Delta Ct}$. $^{*}P < 0.05$, $^{**}P < 0.01$. Gene-expression data of *CCL26, IL5, IL13, PRG2, POSTN,* and *FLG* were used to perform a principal component analysis comparing PPI-R (n = 12) and PPI-NR (n = 9) patients at baseline (G), and PPI-R patients after and before PPI-treatment (H). PPI-NR = proton-pump inhibitor-nonresponders; PPI-R = proton-pump inhibitor-responders.

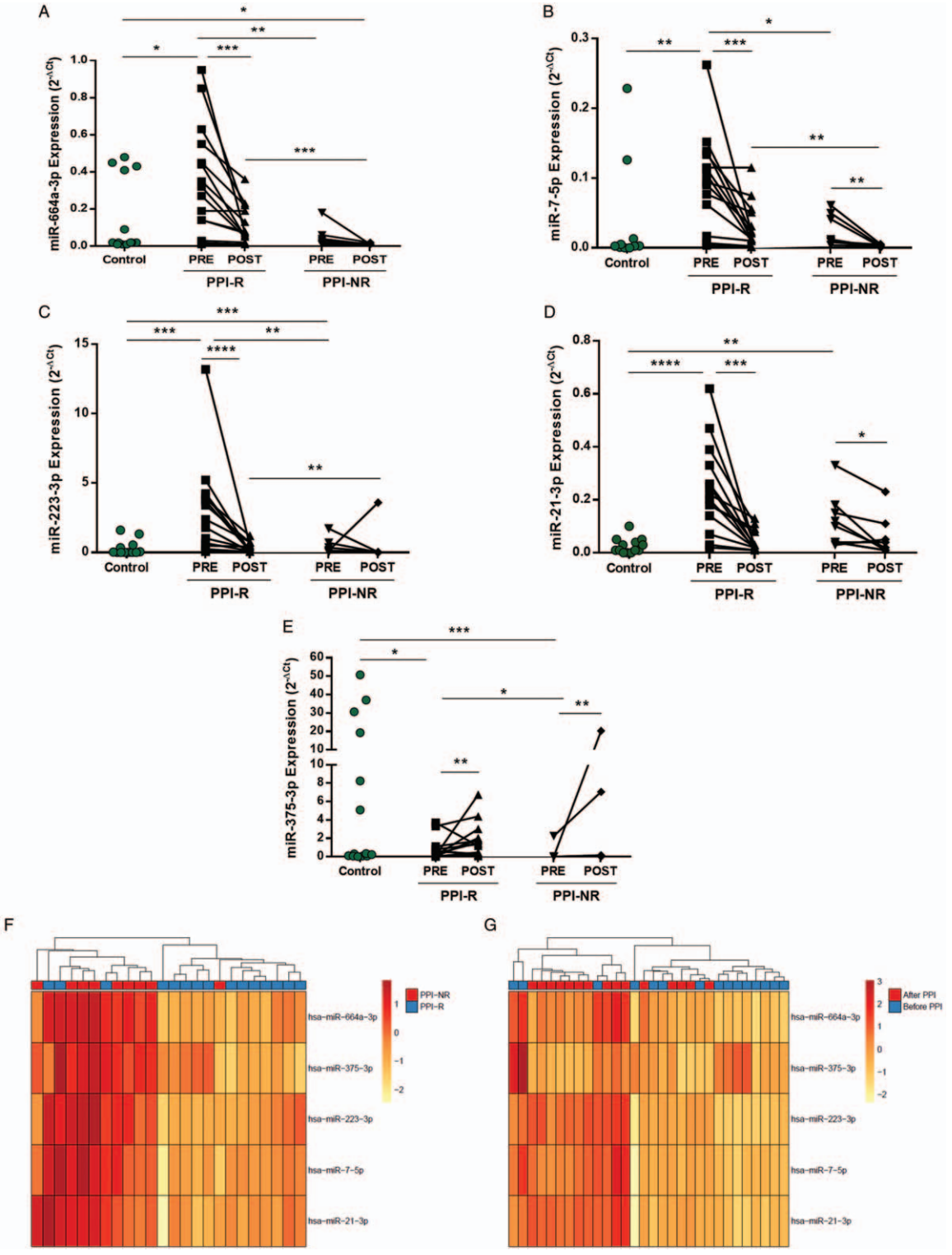

FIGURE 2. MicroRNA expression profile was modified by proton-pump inhibitors and allowed differentiation between proton-pump inhibitor-responder and proton-pump inhibitor-nonresponder patients. Expression of miR-664a-3p, miR-7-5p, miR-223-3p, miR-21-3p, and miR-375-5p (A–E) differs in PPI-R patients before and after treatment. MiRNAs were compared among controls (n = 15), PPI-R children (n = 15), and PPI-NR patients (n = 9) before and after PPI-administration. All experiments were performed in triplicate. Relative miRNA expression is expressed as $2^{-\Delta Ct}$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. Unsupervised hierarchical clustering heatmap analysis was performed with differential miRNAs, which is more suitable to discriminate PPI-R from PPI-NR patients (F and G). miRNA = microRNA; PPI-NR = proton-pump inhibitor-nonresponders; PPI-R = proton-pump inhibitor-responders.

TABLE 2. Logistic regression model for proton-pump inhibitor-responders versus proton-pump inhibitor-nonresponders at baseline

	miRNA	OR	(95% CI)	*P* value	AUC (95% CI)
Logistic regression	miR-7-5p	0.10	0.01–0.65	0.011	0.90 (0.75–1.00)
	miR-375-3p	1.68	1.06–3.17	0.025	
	has-miR-223-3p	7.48	1.64–99.1	0.005	

Individual values of miRNA odds ratio, confidence interval, *P*-value, and area under curve for the combination of 3 miRNAs. AUC = area under curve; CI = confidence interval; miRNA = microRNA; OR = odds ratio.

in these patients. This discovery could be associated with some histologic findings that are also able to differentiate between these patients, such as esophageal eosinophils number. In this sense, we investigated the association between miRNA expression and eosinophil counts found in distal and upper-mid esophageal biopsies in both groups of patients.

When we performed correlation analysis, we found that miR-223-3p ΔCt values were inversely correlated with upper-mid esophageal eosinophils counts in PPI-R patients, in a significant manner (Supplemental Digital Content 7, *http://links.lww.com/MPG/B990*). Namely, when eosinophil number decreased miR-223-3p expression was also reduced (a higher ΔCt implies a lower expression levels).

In a general manner, responder subjects showed a better correlation than nonresponder patients in both upper-mid and distal biopsies, in the 4 miRNAs evaluated (Supplemental Digital Content 7, *http://links.lww.com/MPG/B990*). This finding could be indicative of preclinical response to PPI, associated esophageal miRNAs with eosinophils.

Serum MicroRNAs Are Not Deregulated in Proton-pump Inhibitor-responder and Proton-pump Inhibitor-nonresponder Patients

Finally, in order to use these miRNAs as noninvasive biomarkers, we investigated possible alterations on their expression in serum. Only the miR-233-3p was, however, detected in serum, and unfortunately, we did not observe any changes among different EoE population evaluated (Supplemental Digital Content 8, *http://links.lww.com/MPG/B991*). Exclusively, we found a significant rise in this miRNA between PPI-R patients at baseline of treatment and controls ($P < 0.05$).

DISCUSSION

Our results have identified for the first time several miRNAs that may predict PPI-therapy response at baseline endoscopy, according to their levels of expression. If reproduceable by other, this finding may help alleviate the need for follow-up endoscopies after PPI treatment in patients with these miRNA levels. Moreover, we corroborated the molecular restoration of several abnormal genes' expression and protein levels after PPI therapy.

In relation to therapy, up to 70% of pediatric patients with EoE achieve clinic-pathologic remission on PPI treatment (19,20). Our study demonstrated a 63% rate (17/27) for a PPI having anti-inflammatory actions, as they inhibit Th2 cytokine-induced eotaxin-3 expression in esophageal epithelial cells in EoE patients by blocking binding STAT6 to the eotaxin-3 promoter (21). Recently, 1 important finding about PPI response has been reported by Mougey et al (22). They demonstrated that the pharmacodynamics of PPI was strongly influenced by genetic variants in *CYP2C19* and in *STAT6*. Interaction among these variants produces a synergic effect that influences the pharmacogenetics of PPI therapy, proving that a genetic component plays an important role in the PPI response in pediatric population with EoE.

Despite studies looking at the molecular basis in PPI-R EoE, to date, there are no effective predictive factors of PPI response. In fact, responder and nonresponder patients are endoscopically, histologically, and molecularly indistinguishable. Given this similarity, we have demonstrated that the most important finding concerns the differential levels of miRNA expression in esophageal biopsies from children with EoE. These values of miRNA expression have been found to discriminate between PPI-R and PPI-NR patients at baseline.

Regarding miRNAs, there are few studies about the role miRNAs play in EoE pathology and there are no studies evaluating miRNAs profile before and after PPI therapy (17,18,23–25).

Among the significant miRNAs discovered, we show novel miRNAs that could be implicated in the pathophysiology of EoE, like miR-664a, which has not been previously described. Recently, a study performed by Zhong et al (26) reported that this miRNA is upregulated in epithelial cell lung in COPD patients, associated to inflammatory lung processes, possibly indicating that miR-664a-3p is associated in the inflammatory events that occur in EoE. The specific roles of miR-664a in this pathology is, however, unknown and more studies are necessary.

Other studies have described that miR-223-3p, miR-7-5p, and miR-21-3p were increased in EoE conditions when compared with controls, and/or differentially altered before and after corticosteroid treatment in EoE patients (17,24). As a result, the down-regulation found in these 3 miRNAs in patients after PPI treatment could help resolve the disease as miR-223 and miR-21 regulate pathways are involved in Th2 polarization and eosinophilia regulation (25,27). Also, miR-223-3p has been reported to be upregulated in patients with other gastrointestinal diseases like GERD, indicating its possible role in inflammatory processes (28). Although, miR-7-5p has been found altered in EoE and its dysregulation is maintained despite glucocorticoid treatment, the specific regulator mechanism in EoE has still not been described (25).

Previously, miR-375 has been found in epithelial cells from allergic patients and particularly in EoE, as it could repress IL-13-mediated effects on this disease (18). The miR-375-3p upregulation found in EoE patients after treatment could indicate a restoration of normal activity of epithelial cells, which could be helped by a decrease of IL-13 levels in these patients. This finding, together with the filaggrin increase seen in PPI-R children after treatment, may indicate a significant improvement of the epithelial barrier, recovering its normal status.

Although PPI-R and PPI-NR patients at baseline may be similar clinically and molecularly, the multivariate logistic regression model established in this study suggests its possible use as a biomarker of response to PPI. These data can be relevant in future PPI-therapy approach of EoE, as this is the first time that esophageal miRNAs have been described as a tool to distinguish between these populations.

Additionally, correlation analysis between miRNA expression and esophageal eosinophil counts could explain more

specifically the response development to therapy. In general, almost all evaluated miRNAs in PPI-R patients tends to decrease with eosinophilia reduction. In PPI-NR, miRNAs, however, did not seem to follow an established pattern. Specifically, we found that miR-223-3p correlated with the degree of tissue eosinophilia of PPI-R children, and this correlation was different from PPI-NR patients. Potentially, this correlation could be indicating the disease severity as previously was reported in patients treated with glucocorticoids (25). In view of our results, however, this miRNA could be a key factor in the prediction of PPI response. Nevertheless, more studies in a higher cohort of patients are necessary.

In order to try and establish novel systemic biomarkers for noninvasive monitory of EoE, our study constitutes an evaluation of these miRNAs in serum. Almost all miRNAs, however, cannot be detected in serum, and the only 1 found, miR-223-3p, did not show significant alterations among the 2 populations analyzed. Previous studies have attempted to search for serum biomarkers to diagnosis EoE without any satisfactory result (29). Consequently, a search of less invasive and novel biomarkers continues to be necessary to predict PPI-therapy response.

Although this study was unable to find a noninvasive way to identify PPI-R from PPI-NR patients, our research used multiple techniques to demonstrate and confirm a wide range of molecular changes in esophageal biopsies from children with EoE after esomeprazole administration. Our results demonstrate this molecular characteristic supporting and consistent with other studies, which have reported that PPI therapy restores esophageal mucosal integrity, reduces Th2 inflammation, and reverses almost all of the EoE transcriptome in responder patients (30,31).

Interestingly, we can speculate that miRNA expression level may be more helpful in following-up the patients who continue to have EoE symptoms despite normal histopathology. Further evaluation of these miRNA expression levels may explain such ongoing symptoms from active biomolecular mechanisms, being this information supportive before getting started on treatments directed towards food protein antigen-induced EoE or corticosteroids.

Despite the important findings found in this study, there are some limitations. First, our studied patients are children from 2 hospitals and the results could be limited to a pediatric-only population, so may not be applicable to adults. Second, this study is of a small sample size; however, the results and differences between the groups were found to be significant. Although differential values of miRNA expression have been found to distinguish PPI-R and PPI-NR, more studies are necessary to confirm this finding on a larger scale as well as to identify noninvasive serum biomarkers.

CONCLUSIONS

In conclusion, we demonstrate the existence of miRNA expression levels in esophageal biopsies from children with EoE treated with esomeprazole. They can discriminate between PPI-R and PPI-NR patients at baseline being prognostic indicators and can monitor treatment response in subjects who respond to PPI.

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