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ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

Proton pump inhibitor effect on esophageal protein signature of eosinophilic esophagitis, prediction, and evaluation of treatment response

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Abstract

Background: Recently, we have identified a dysregulated protein signature in the esophageal epithelium of eosinophilic esophagitis (EoE) patients including proteins associated with inflammation and epithelial barrier function; however, the effect of proton pump inhibitor (PPI) treatment on this signature is unknown. Herein, we used a proteomic approach to investigate: (1) whether PPI treatment alters the esophageal epithelium protein profile observed in EoE patients and (2) whether the protein signature at baseline predicts PPI response.

Abbreviations: EoE, eosinophilic esophagitis; GERD, gastroesophageal reflux disease; hpf, high-power field; IPA, Ingenuity Pathway Analysis; PCA, principal component analysis; PostPPI, after PPI treatment; PrePPI, before PPI treatment; PPI, proton pump inhibitors; PPI-REE, proton pump inhibitor-responsive esophageal eosinophilia; RNAseq, RNA sequencing.

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Methods: We evaluated the protein signature of esophageal biopsies using a cohort of adult EoE ($n = 25$) patients and healthy controls (C) ($n = 10$). In EoE patients, esophageal biopsies were taken before (pre) and after (post) an 8-week PPI treatment, determining the histologic response. Eosinophil count PostPPI was used to classify the patients: ≥15 eosinophils/hpf as non-responders (non-responder) and < 15 eosinophils/hpf as responders (R). Protein signature was determined and differentially accumulated proteins were characterized to identify altered biological processes and signaling pathways.

Results: Comparative analysis of differentially accumulated proteins between groups revealed common signatures between three groups of patients with inflammation (responder-PrePPI, non-responder-PrePPI, and non-responder-PostPPI) and without inflammation (controls and responder-PostPPI). PPI therapy almost reversed the EoE specific esophageal protein signature, which is enriched in pathways associated with inflammation and epithelial barrier function, in responder-PostPPI. Furthermore, we identified a set of candidate proteins to differentiate responder-PrePPI and non-responder-PrePPI EoE patients before treatment.

Conclusion: These findings provide evidence that PPI therapy reverses the alterations in esophageal inflammatory and epithelial proteins characterizing EoE, thereby providing new insights into the mechanism of PPI clinical response. Interestingly, our results also suggest that PPI response could be predicted at baseline in EoE.

KEYWORDS
eosinophilic esophagitis, prediction treatment response, protein signature, proton pump inhibitors

1 | INTRODUCTION

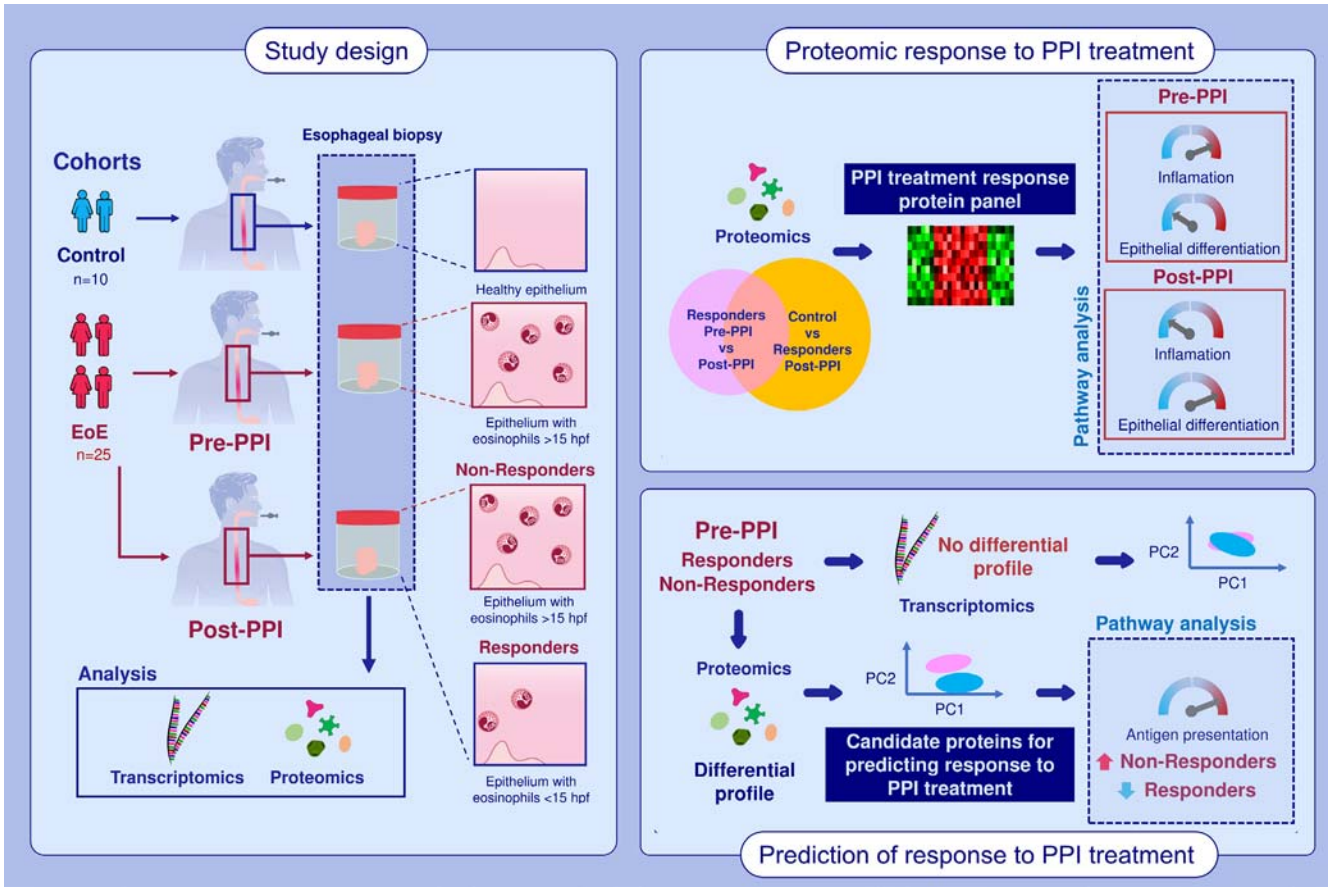
Eosinophilic esophagitis (EoE) is a chronic, immune-mediated inflammatory disease that is characterized by esophageal dysfunction and infiltration of the esophagus by eosinophils.[1–3]

Eosinophil infiltration in the esophageal mucosa of over 15 cells per high-power field (hpf) are the cut-off used for diagnosis.[4,5] Epithelial injury is commonly observed in EoE biopsies, together, hyperplasia of the basal layer, tissue regeneration, and fibrosis in the lamina propria.[6] The natural course of EoE is chronic and apparently progressive, with continuous inflammation that may progress to tissue remodeling thus leading complications such as esophageal narrowing and dysmotility in the long term.[2,3] Therefore, keeping the disease in remission by controlling esophageal inflammation is key for the long-term management of EoE patients.[4,5] Notably, esophageal remodeling does not correlate well with epithelial eosinophil counts, and inflammatory activity usually does not associate to patients' symptoms.[7]

The pathophysiology underlying EoE is complex and involves environmental and immunologic determinants along with genetic susceptibility loci.[8,9] EoE is characterized by increased levels of type 2 cytokines, such as interleukin (IL)-4, IL-5, IL-13, and thymic stromal lymphopoietin (TSLP) as well as eosinophil chemotactic proteins such as eotaxin-3.[8,9] These cytokines are critical for promoting cellular responses in EoE, including eosinophil recruitment and activation, epithelial barrier dysfunction, tissue remodeling, and eventually fibrosis. Barrier dysfunction of the esophageal epithelium, resulting in the loss of proteins such as desmoglein-1 (DSG1), filaggrin (FLG) and involucrin (IVL), alters defensive mechanisms allowing allergen penetration, and promoting recruitment and activation of immune cells, thereby perpetuating the inflammatory response.[10]

Over the last decade, clinical practice guidelines have provided an evidence-based framework to manage patients with EoE, which proton pump inhibitor (PPI), topical steroids, and dietary modifications as first-line therapies.[4,5] Patients with fibrotic strictures or narrow-caliber esophagus should be assessed with endoscopic dilation.[4,5] Furthermore, high patient relapse rates, and the associated need for long-term therapies have promoted the search for novel EoE treatments including antibody-based therapies targeting inflammatory type 2-related molecules, such as against IL-4, IL-13, and TSLP, among others.[11]

PPIs, omeprazole and derivatives, are the most commonly prescribed first-line therapy for EoE because of their efficacy, safety profile, easy administration, and low cost.[12] Their efficacy in EoE has been reported by multiple studies to be in the range of 33% to 50% depending on the criteria used to define histologic remission.[13] Several studies suggest that the underlying mechanism for the efficacy of PPIs in EoE depends on their anti-inflammatory effect



GRAPHICAL ABSTRACT

Proteomics and transcriptomics were used to investigate changes in esophageal epithelium from EoE responder and non-responder patients prior and after PPI treatment. A set of proteins, mainly related to inflammatory and epithelial differentiation processes, were detected as differentially expressed in PPI responders after treatment. Proteins related to antigen presentation were differentially expressed between responders and non-responders at baseline.

Abbreviations: EoE, eosinophilic esophagitis; hpf, high-power field; mRNA, messenger RNA; PostPPI, after PPI treatment; PrePPI, before PPI treatment; PC, principal component; PPI, proton pump inhibitors.

and even on their acid suppression capacities.[12] These dual effects of PPI could contribute to resolution of esophageal eosinophilia in both GERD and EoE. In addition, PPI therapy partially restores epithelial barrier integrity in PPI responders' patients, contributing to its effect on EoE.[14] Mechanistically, PPI therapy partially restores mucosal integrity in patients with PPE.PPIs downregulate expression of eotaxin-3 and other Th2-dependent cytokines in the esophageal mucosa.[15] Additionally, PPI offer a direct anti-inflammatory effect through blockade of STAT6 binding to eotaxin-3 promoter.[16] Another effect of PPIs on esophageal epithelium is the modulation of IL-13–induced responses, highlighting the importance of aryl hydrocarbon receptor in these processes.[17]

Currently, predictive markers of response remain to be identified. In early times, a consensus recommendation for EoE diagnosis postulated trying PPI to exclude gastroesophageal reflux disease (GERD), since this entity can elicit an acid-induced esophageal eosinophilia.[18,19] However, currently, EoE and PPI-responsive esophageal eosinophilia (PPI-REE) are considered as variations of a single disease and a PPI response is no longer considered as a diagnostic criterion in EoE.[20] This consensus has been consolidated since clinical characteristics, endoscopic and histologic findings, pH monitoring, and tissue/genetic markers, including DNA, mRNA, and proteins as such IL5, eotaxin-3, and filaggrin, have failed to distinguish EoE from PPI-REE.[12] In particular, a well stablished gene-based EoE diagnostic panel, the EDP panel, has demonstrated that untreated PPI-responders share a largely similar molecular transcriptome with non-responders.[21] Alternatively, several microRNAs in esophageal biopsies might discriminate between PPI responders and non-responders at baseline.[22] Recently, an RNAseq analysis in esophageal biopsies found no differentially expressed individual genes predicting topical corticosteroid response in EoE. Interestingly, a 22 gene panel was associated with subsequent histologic response to corticoids, although this result was not validated in an independent cohort.[23]

Genetic determinants that influence response to PPI such as pharmacogenetic variants in STAT6 and CYP2C19 (a transcription factor and a PPI metabolizer, respectively), have been described.[24] Other genetic variations also involved in PPI metabolism may explain those cases not related to CYP2C19; drugs, environmental and dietary factors acting as inhibitors or inducers of this enzyme activity should be also considered.[25]

We have recently described the usefulness of an EoE associated protein signature.[26] Furthermore, Rochman et al.,[27] confirmed that immune-related proteins were upregulated, whereas proteins linked to epithelial differentiation were primarily downregulated in esophageal biopsies from EoE patients. Additionally, authors showed that all 6 protein subunits of the minichromosome maintenance (MCM) complex, a DNA helicase essential for genomic DNA replication, were upregulated, suggesting that MCM complex inhibition could be used to block the esophageal epithelial proliferation observed in EoE. In addition, it has been determined that fibroblasts from patients with EoE secrete a unique extracellular matrix proteome that shifts normal esophageal fibroblast toward a profibrotic phenotype.[28]

Herein, we hypothesized that the differences observed in protein accumulation, including inflammatory-and epithelial-related proteins, between esophageal biopsies from controls and EoE patients.[26] might be altered by PPI treatment. Furthermore, protein signature analysis could distinguish between responders and non-responders before PPI treatment, helping in the management of EoE patients.

2 | MATERIALS AND METHODS

2.1 | Subjects

Adult EoE patients were prospectively recruited at two Spanish hospitals, Hospital Universitario de La Princesa (Madrid), and Hospital General de Tomelloso (Ciudad Real) between February 2018 and November 2020.[26] EoE was diagnosed according to evidence-based guidelines.[4,5] For EoE diagnosis purposes, three esophageal biopsies were obtained for each patient at the distal and proximal esophagus.[4,5] Esophageal eosinophilia was defined as an eosinophil count of ≥15 cells per hpf (corresponding to an area of 0.24 mm_2) in one or more biopsy specimens. The Dysphagia Symptoms Score (DSS) was used for clinical assessment.[29] EoE patients underwent an 8-week course of PPI therapy (20–40 mg of omeprazole or equivalent doses of available PPI agents (BID twice daily)) and after that, esophageal biopsies were taken. Recent evidence has been provided recently that 40 mg of omeprazole BID was not superior to 20 mg BID in achieving histologic remission of EoE, and both doses represented the most effective approach for adult patients.[30] PPI response was defined as positive when the post-treatment esophageal eosinophilia was resolved (responder-PostPPI, $N = 14$) (<15 eosinophils/hpf). After histologic evaluation, all non-responder-PostPPI patients ($N = 11$) were defined as those patients who remained with a peak eosinophil count over 15 cells per hpf (the diagnostic threshold for EoE) after PPI therapy, according to standard definitions of response in a clinical practice setting.[31]

Controls were subjects who underwent upper endoscopy for assessment of dyspepsia or suspected gastroduodenal ulcer; no patient was under PPI therapy at the time of endoscopy. All selected control subjects exhibited a normal endoscopic appearance of the esophagus and they did not meet clinical or histological criteria for EoE after endoscopy and biopsy. Clinical data including demographics, symptoms, atopic background and endoscopic findings were recorded from all EoE patients and control subjects.

This study (PI17/0008) was approved by the Research Ethics Committee of Instituto de Investigación Sanitaria Hospital Universitario de La Princesa (registry number 3107, 8 June 2017). All patients and controls signed an informed consent form before sampling.

2.2 | Esophageal biopsies processing, mass spectrometry, RNAseq, and bioinformatics and statistical analysis

A detailed description of the methods used has been included in the supplementary Material and Methods section—Data S1. Information of the antibodies employed is summarized in the Table S1.

3 | RESULTS

3.1 | Characteristics of EoE and control patients

Clinical and demographic characteristics of study participants are summarized in Table 1.

Of the 25 EoE patients, 14 (56%) were classified as PPI-responders (R) and 11 (44%) as PPI-non-responders (non-responder) (Table 1). Compared with controls ($n = 10$) and responders, non-responder patients were older (32 and 35.92 vs. 46.63 years, respectively), although significant differences were only found between control and non-responders. Non-responders were more frequently male compared to control and responders (100% vs. 60% and 78%), and significant differences were only found between non-responders and the control group. Of note, there were no differences in demographic variables between EoE groups. There were no significant differences regarding symptoms, (DSS score[29]), endoscopic (EREFS score[32] and erosive esophagitis), and histological (EoE-HSS[33]) findings, or PPI treatment options at baseline. The PPI therapy lowered eosinophil count to <15 cells/hpf in all PPI responder cases, reducing the mean peak eosinophil count significantly from 53.64 to 2.28 ($p < 0.001$) (Table 1).

3.2 | Comparative proteomic profile in esophageal biopsies of responder and non-responder EoE patients compared to controls

We have recently determined a specific proteomic profile of esophageal biopsies associated to EoE.[26] To address whether PPI-treatment promotes changes in this EoE-associated protein signature, we evaluated the global proteomic profile in esophageal

TABLE 1 Characteristics of control and EoE responders and non-responders patients.

	Control ($n=10$)	Responders ($n=14$)	Non-responders ($n=11$)	*p-value* (C vs. R)	*p-value* (C vs. N)	*p-value* (R vs. N)
Sex (male) (*n*, %)	6 (60%)	11 (78%)	11 (100%)	0.392	**0.035**	0.23
Age (mean years ± SD)	32 ± 11.6	35.92 ± 11.75	46.63 ± 14.05	0.427	**0.017**	0.056
Symptoms (*n*, %)						
Dysphagia	0	13 (93%)	11 (100%)	**0.002**	**<0.001**	1
Food impaction	0	11 (78%)	6 (55%)	**0.001**	**0.012**	0.389
Heartburn	0	4 (28%)	4 (36%)	0.114	0.090	1
Abdominal pain	0	0 (0%)	2 (18%)	1	0.476	0.183
DSS	–	9.14 ± 3.70	8.73 ± 3.26	–	–	0.768
≥8	–	10 (71%)	8 (73%)	–	–	1
Any atopic disease (*n*,%)						
Asthma	0	7 (50%)	1 (0.9%)	**0.018**	1	**0.042**
Allergic rhinitis/sinusitis	1 (10%)	11 (78%)	10 (90%)	**0.004**	**<0.001**	0.604
Food allergy	2 (20%)	6 (43%)	1 (0.9%)	0.387	0.586	0.09
Endoscopic findings (*n*, %)						
Edema	0	12 (86%)	9 (81%)	**<0.001**	**<0.001**	1
Rings	0	6 (43%)	7 (63%)	0.081	**0.006**	0.428
Exudates	0	9 (64%)	9 (81%)	**0.002**	**<0.001**	0.407
Furrows	0	11 (78%)	9 (81%)	**<0.001**	**<0.001**	1
Stricture	0	2 (14%)	3 (27%)	0.492	0.214	0.623
Narrowing	0	3 (21%)	2 (18%)	0.239	0.476	1
EREFS (score) (mean ± SD)	0	3.5 ± 1.9	4.3 ± 1.6	**<0.001**	**<0.001**	0.285
Erosive esophagitis	0	4 (28%)	4 (36%)	0.114	0.09	1
Histological findings						
Maximum eosinophil count (mean ± SD)	0	53.6 ± 31.6	61.6 ± 21.4	**<0.001**	**<0.001**	0.46
Eosinophil count post PPI (mean ± SD)	-	2.28 ± 3.49	85.54 ± 66.79	–	–	**0.002**
EoEHSS GRADE score (0–1) (mean ± SD)	0	0.48 ± 0.18	0.49 ± 0.21	**<0.001**	**0.001**	0.866
Eosinophil density	0	9 (64%)	11 (100%)	**<0.001**	**<0.001**	0.343
Basal zone hyperplasia	0	11 (78%)	9 (81%)	**<0.001**	**<0.001**	1
Eosinophil abscesses	0	9 (64%)	7 (64%)	**0.002**	**0.004**	1
Eosinophil surface layering	0	6 (43%)	6 (54%)	**0.024**	**0.012**	0.695
Dilated intercellular spaces	0	14 (100%)	10 (90%)	**<0.001**	**<0.001**	0.44
Surface epithelial alteration	0	11 (78%)	7 (64%)	**<0.001**	**0.004**	0.695
Dyskeratotic epithelial cells	0	3 (21%)	4 (36%)	0.239	0.090	0.695
EoEHSS stage score (0–1) (mean ± SD)	0	0.45 ± 0.17	0.42 ± 0.21	**<0.001**	**<0.001**	0.751
Eosinophil density	0	14 (100%)	10 (90%)	**<0.001**	**<0.001**	0.922
Basal zone hyperplasia	0	11 (78%)	9 (81%)	**<0.001**	**<0.001**	1
Eosinophil abcesses	0	9 (64%)	7 (64%)	**0.002**	**0.004**	1
Eosinophil surface layering	0	6 (43%)	6 (54%)	**0.024**	**0.012**	0.695
Dilated intercellular spaces	0	14 (100%)	10 (90%)	**<0.001**	**<0.001**	0.44
Surface epithelial alteration	0	11 (78%)	7 (64%)	**<0.001**	**0.004**	0.695
Dyskeratotic epithelial cells	0	3 (21%)	4 (36%)	0.239	0.090	0.695

Note: Clinical characteristics of EoE patients before PPI treatment (responders and non-responders) and healthy controls (C). Fisher's test and Student's *t*-test were used to analyze significant demographics and clinical differences between groups, *p*-value is indicated. Bold *p* values are less than 0.05.

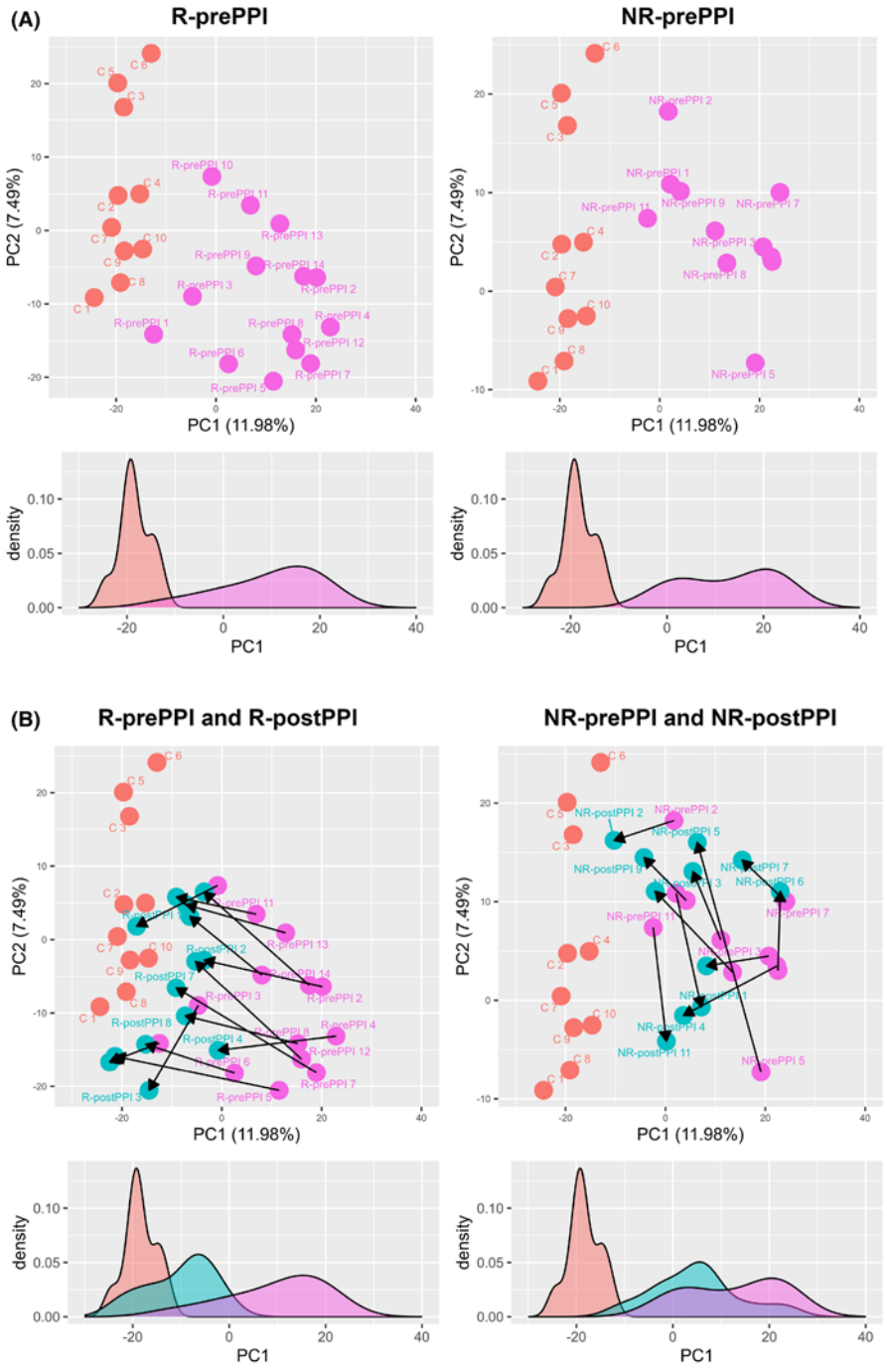


FIGURE 1 Comparative protein expression in esophageal biopsies from EoE patients. (A) PCA analysis of whole proteomic data. PC1 and PC2 are represented and the percentage of explained variance is indicated on each axis. The samples are colored by group: A; control (red), responder-PrePPI (pink, left), non-responder-PrePPI (pink, right). (B) Control (red), responder-PrePPI (pink, left), responder-PostPPI (green, left), non-responder-PrePPI (pink, right), non-responder-PostPPI (green, right). Additionally, PC1 density was plotted (A and B, bottom panels). In paired samples, arrows indicate movement of EoE patients after PPI treatment.

biopsies from both R and non-responder before and after PPI intake. As illustrated by a principal component analysis (PCA) of the global proteomic profile (Figure 1A) clear separation between control and EoE patients prior PPI (including responder-PrePPI and non-responder-PrePPI) treatment was observed across the first principal component, which accounted for 11.98% of total sample variance (Figure 1A, upper lane). When the expression profiles were plotted according to the first principal component density, responder-PrePPI and non-responder-PrePPI groups showed comparable distribution patterns (Figure 1A, lower lane), thus suggesting that a common EoE protein signature pattern existed, independently of PPI response capacity.

To address whether PPI treatment altered the EoE global protein signature we compared the responders and non-responder cohorts before and after PPI treatment (responder-PrePPI vs. responder-PostPPI and non-responder-PrePPI vs. non-responder-PostPPI) (Figure 1B). Graphical representation of PCA components 1 and 2 suggests that PPI therapy is effective at reducing, even eliminating in some patients, differences between responder-PostPPI and controls associated proteomic profiles, with a clear movement to the left side of the plot. Of note, a modest effect with similar characteristics was also observed in non-responder-PostPPI patients suggesting that EoE proteomic profile was partially abrogated in non-responder patients, despite their histologic

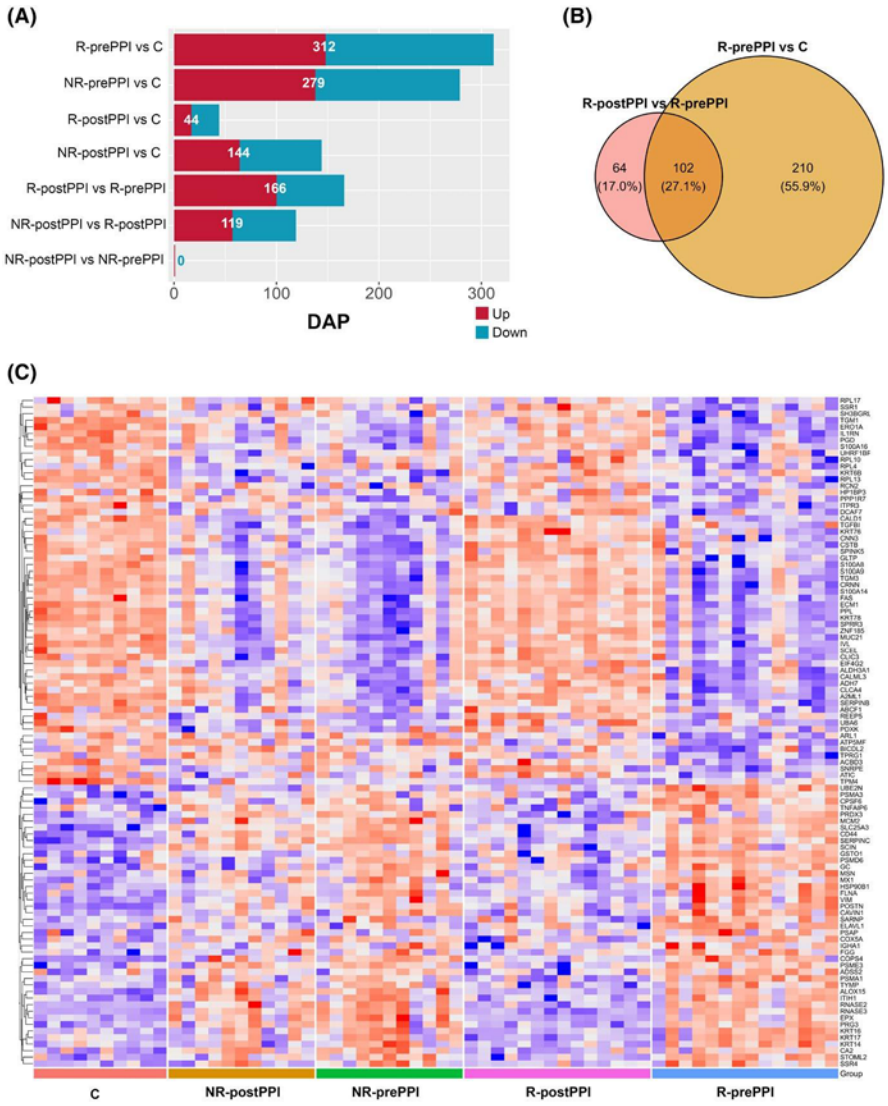


FIGURE 2 Comparative protein expression profiles of study cohorts. (A) Differentially accumulated proteins (adjusted *p*-value ≤0.05 and a 1.5-fold change) in esophageal biopsies obtained by comparisons of control and EoE cohorts (controls $n = 10$; responder-PrePPI $n = 14$; non-responder-PrePPI $n = 11$; responder-PostPPI $n = 14$; non-responder-PostPPI $n = 11$). Red indicates upregulated differentially accumulated proteins, and blue, downregulated differentially accumulated proteins relative to the second group in the comparison. Number of differentially accumulated proteins is indicated. (B) Venn diagrams showing the overlap between dysregulated proteins in both controls versus responder-PrePPI, and responder-PrePPI versus responder-PostPPI C. Heatmap of the 102 proteins selected in panel B in the 5 cohorts. z-score indicating protein levels is presented. Red indicates higher expression (upregulation), and blue represents lower expression (downregulation).

eosinophil-related response was null or incomplete (Figure 1B). These results were clearly illustrated when the expression profiles were plotted according to the first component density (Figure 1B, lower lane). Overall, data confirmed that global protein signature from esophageal biopsies could be informative to molecularly define PPI response in EoE patients.

3.3 | Effect of PPI treatment on the protein signature of EoE

It has been extensively reported that after PPI therapy, histologic eosinophilia remission is associated with a specific mRNA-signature,[21] but it is unknown whether EoE-associated protein signature recover control values after PPI therapy. To address this, we evaluated protein levels in esophageal biopsies from EoE patients before and after PPI treatment. Differentially accumulated proteins in esophageal biopsies were identified after comparison between control individuals and all groups of EoE patients (Figure 2A and Tables S2–S5). Comparative analysis (adjusted *p*-value ≤0.05 and fold change>1.5) showed the following results related to differentially accumulated proteins: responder-PrePPI versus controls (312 proteins, 148 upregulated, 164 downregulated); responder-PostPPI versus controls (44 proteins, 17 up, 27 down); non-responder-PrePPI versus controls (279 proteins, 138 up, 141 down); non-responder-PostPPI versus controls (144 proteins, 64 up, 80 down).

Next, we compared responder patients before and after treatment responder-PostPPI versus responder-PrePPI (166 proteins, 100 upregulated and 66 downregulated) and non-responder-PostPPI versus non-responder-PrePPI (none). Finally, non-responder-PostPPI were compared to responder-Post-PPI (119 proteins, 57 upregulated and 62 downregulated). A complete information regarding differentially accumulated proteins obtained in all comparisons is included in Tables S6–S8.

To identify a protein signature associated with PPI response, common differentially accumulated proteins in controls versus responder-PrePPI and responder-PrePPI versus responder-PostPPI comparisons were analyzed (Figure 2B; Tables S2 and S6). Results showed 102 differentially accumulated proteins shared by both comparisons (Table S9). Next, we evaluated the expression profile of these 102 differentially

accumulated proteins in all five groups (Figure 2C). As illustrated by the supervised clustered heatmap, there was a remarkable conservation of the esophageal epithelium proteomic profile between controls and responder-PostPPI groups (Figure 2C). In contrast, this pattern was different in the pretreatment groups (responder-PrePPI, non-responder-PrePPI) and post-treatment non-responder-PostPPI group. responder-PostPPI samples showed similar levels to control samples probably associated with the lower inflammatory state of these patients. In contrast, groups with esophageal eosinophilia (responder-PrePPI, non-responder-PrePPI and non-responder-PostPPI) showed more similar patterns between them but different to control and responder-PrePPI patients. This protein panel associated to PPI-response included eosinophil-related proteins not detected at mRNA level (ribonuclease A family member 2, RNAse2; ribonuclease A family member 3, RNAse3; eosinophil peroxidase, EPX), and 32 esophagus-enriched genes[34] (Table S9) strongly suggesting that altered pathways in EoE[26] related to inflammation and epithelial differentiation are recovered in responder-PostPPI patients.

Next, we annotated these proteins using the Gene Ontology (GO) database. Out of all of them, 78 proteins were categorized as intracellular, 45 were designated as membranous, and 11 were identified as secreted proteins (Figure S1, Panel A). Reflecting the intricate nature of protein biology, several proteins were found to possess multiple annotations, being labeled *simultaneously* as both intracellular/membrane or secreted proteins. Theoretically, secreted proteins could be measured using minimally invasive methods, such as blood tests, or through devices such as the String test.[35]

Finally, to further confirm these observations we validated the expression of two selected proteins by immunofluorescence analyses in representative tissue samples (responder-PrePPI and non-responder-PrePPI EoE patients): histocompatibility minor 13, HM13, and protein phosphatase methylesterase 1, PPME1 (Figure S2).

3.4 | Immunofluorescence-based assessment of protein expression confirms proteomic data

Next, we validated the expression of several differentially accumulated proteins that are representative of the inflammatory and epithelial alterations observed between control and the different EoE cohorts by immunofluorescence in esophageal biopsies. We selected two proteins that were significantly upregulated in EoE patients with inflammation (responders-PrePPI, non-responder-PrePPI, and nonresponder-PostPPI) comparing them to those without inflammation or minimally inflamed (controls, responders-PostPPI) (CD44; Arachidonate 15-Lipoxygenase, ALOX15). In addition, we analized two proteins that were significantly downregulated (cornulin, CRNN; mucin21, MUC21) in inflamed samples. Immunofluorescence analysis showed similar results to proteomic data highlighting that an effective PPI treatment reverse the EoE-associated protein signature (Figure 3).

3.5 | In depth protein signature analysis after PPI treatment

To thoroughly characterize the effect of PPI on esophageal biopsies, we evaluated the protein profile in paired samples from responder patients before and after treatment with PPI. In this cohort, 8 weeks of PPI therapy reduced the mean peak eosinophil count significantly from 53.64 to 2.28 ($p < 0.001$), as well as the symptomatic-related DSS score from 9.14 to 4.86 ($p < 0.01$), EREFS score, from 3.5 to 1.41 ($p < 0.001$) and the EoE HSS score from 0.48 to 0.04 ($p < 0.001$) (Table 2). On the other hand, non-responding patients showed no significant changes in their eosinophil peak count, and symptomatic, endoscopic and histological scores in comparison to baseline values (Table 2).

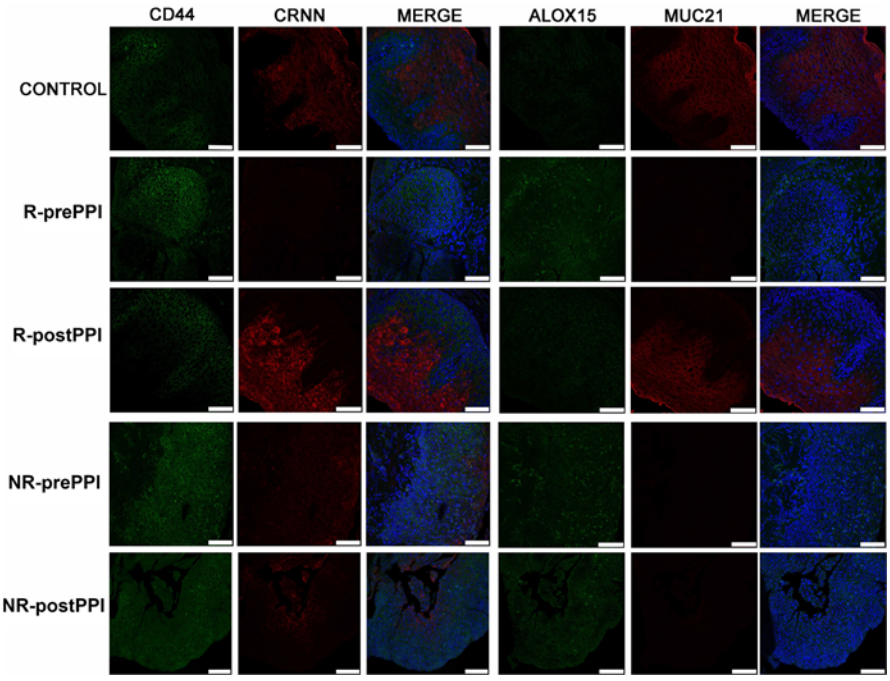


FIGURE 3 Expression of selected differentially accumulated proteins in esophageal biopsies from PPI responder EoE patients. Immunofluorescence analysis of CD44, CRNN, ALOX15 and MUC21 in esophageal biopsy sections from a presentative control subject and representative responder-PrePPI, responder-PostPPI, non-responder-PrePPI and non-responder-PostPPI EoE patients. Serial tissue sections were stained with pairs of antibodies to simultaneously detect: CD44 (green) and CRNN (red); ALOX15 (green) and MUC21 (red). Differentially accumulated proteinsl (blue) is shown in merge images. Scale bar 75 μm. Images are representative of each group.

TABLE 2 Characteristics of EoE responders and non-responders patients before and after treatment with PPI.

	R-PrePPI ($n=14$)	R-PostPPI ($n=14$)	NR-PrePPI ($n=11$)	NR-PostPPI ($n=11$)	p-value (R-PrePPI vs. R-PostPPI)	p-value (NR-PrePPI vs. NR-PostPPI)
Symptoms (n, %)						
Dysphagia	13 (93%)	8 (57%)	11 (100%)	10 (91%)	0.077	1
Food impaction	11 (79%)	2 (14%)	6 (55%)	2 (14%)	**0.002**	0.387
Heartburn	4 (29%)	0 (0%)	4 (36%)	0 (0%)	0.098	0.090
Abdominal pain	0 (0%)	0 (0%)	2 (18%)	0 (0%)	1	0.476
DSS	9.14 ± 3.70	4.86 ± 3.01	8.73 ± 3.26	6.55 ± 4.30	**0.007**	0.173
≥8	10 (71%)	3 (21%)	8 (73%)	5 (45%)	**0.021**	0.387
Endoscopic findings (n, %)						
Edema	12 (86%)	3 (21%)	9 (81%)	7 (50%)	**0.002**	0.805
Rings	6 (43%)	6 (43%)	7 (63%)	9 (81%)	1	0.913
Exudates	9 (64%)	2 (14%)	9 (81%)	7 (63%)	**0.018**	0.842
Furrows	11 (78%)	3 (21%)	9 (81%)	6 (54%)	**0.010**	0.361
Stricture	2 (14%)	0 (0%)	3 (27%)	4 (36%)	0.481	1
Narrowing	3 (21%)	1 (7%)	2 (18%)	1 (9%)	0.595	1
EREFS score (mean ± SD)	3.5 ± 1.91	1.41 ± 1.29	4.27 ± 1.61	4 ± 2.28	**0.003**	0.67
Histological findings						
Maximum eosinophil count (mean ± SD)	53.64 ± 31.57	2.28 ± 3.49	61.63 ± 21.4	85.54 ± 66.8	**<0.001**	0.272
EoEHSS grade score (0–1) (mean ± SD)	0.48 ± 0.18	0.04 ± 0.04	0.49 ± 0.21	0.37 ± 0.21	**<0.001**	0.184
Hyperplasia basal zone	11 (78%)	0 (0%)	9 (81%)	8 (72%)	**<0.001**	0.296
Eosinophil abcess	9 (63%)	0 (0%)	7 (63%)	4 (36%)	**<0.001**	0.587
Intercellular spaces	14 (100%)	2 (14%)	10 (90%)	11 (100%)	**<0.001**	0.521
Dyskeratotic epithelial cells	3 (21%)	1 (7%)	4 (36%)	3 (27%)	0.596	0.635
EoEHSS stage score (0–1) (mean ± SD)	0.45 ± 0.17	0.01 ± 0.03	0.42 ± 0.21	0.31 ± 0.21	**<0.001**	0.227

Note: Clinical characteristics of EoE responder and non-responder patients before and after PPI treatment. Fisher's test and Student's t-test were used to analyze significant clinical differences between groups, p-value is indicated.

A total of 166 proteins were identified as differentially accumulated when comparing responder patients before and after treatment (Figure 4A, left). Specifically, 100 proteins were upregulated and 66 were downregulated (Table S6). In addition, a heatmap was generated using these proteins from the previous analysis (Figure 4B) clearly showing a different protein accumulation profile. On the contrary, non-responding patients when compared before and after treatment did not present a substantial variation in their proteomic profile and no differentially accumulated proteins were found (Figure 2A).

To identify biological processes and pathways in which the differentially accumulated proteins (with p-value ≤0.05 logFC>1.5) in PPI-responder patients are involved, we performed a gene enrichment analysis using String software tool (https://string-db.org/),[36] using adjusted p-value ≤0.05. Immune activation-related pathways, including neutrophil degranulation, were significantly enriched in upregulated proteins in responding PrePPI patients (adjusted p-value $<10^{-11}$) (Figure 4C). Proteins associated with vesicle mediated transport were also upregulate, suggesting an active vesicle trafficking in the damaged areas. In the downregulated protein subset, we identified cornification and keratinization as the most enriched biological processes (adjusted p-value $<10^{-9}$ and $<10^{-14}$, respectively) (Figure 4C).

Remarkably, we found that several differentially accumulated proteins between responder-PostPPI and responder-PrePPI were undetectable at mRNA level in our previous analysis (Table S10). Applying a selective criterion (fold change>1.5 adjusted p-value ≤0.05) and focusing on the most relevant proteins in EoE three main groups of proteins could be observed (Table S10). The first includes four eosinophil granule-derived proteins, proteoglycan 3 (PRG3), ribonuclease A family member 3 (RNASE3), eosinophil peroxidase (EPX), and RNASE2. A second group including Inter-alpha-trypsin inhibitor heavy chain (ITIH1), serpin family C member 1 (SERPINC1), and microsomal triglyceride transfer protein (MTTP) are typically synthesized in the liver with a constitutive secretion into blood.[34] The third, a miscellaneous group, includes a keratin (KRTN-76) and myosin heavy chain 7B (MYH7), which are also expressed by basophils, according to the Human Protein Atlas database[34] and were previously detected in the esophageal mucosa by a global human tissue proteomic iniciative.[37]

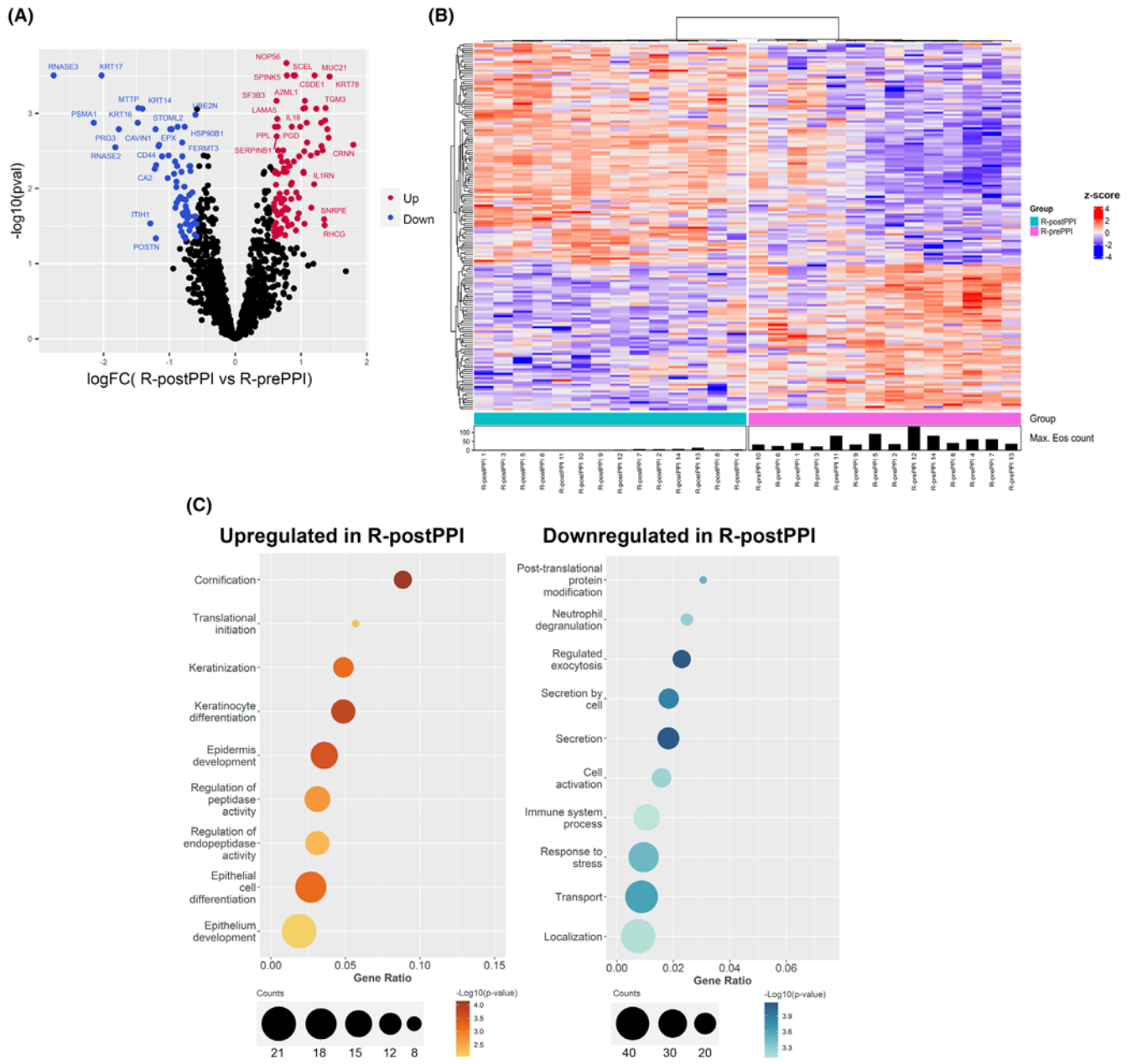


FIGURE 4 Comparative protein expression in PPI response. (A) Volcano plot representation of the differential expression analysis. Log2 fold change is represented on the *x* axis, and −log10 of adjusted *p*-values on the *y* axis. The proteins are colored by relative expression values: Upregulated or downregulated (red and blue, respectively) in responder-PostPPI. Differential expression criteria are an adjusted *p*-value ≤0.05 and a 1.5-fold change. Gene names are shown for the most extreme values. (B) Heatmap showing Z-score scaled protein expression of 166 differentially expressed proteins between responder-PostPPI and responder-PrePPI patients. Sample group is indicated, with the maximum eosinophil count from each subject. The proteins are hierarchically clustered using Euclidean distance, as shown in the top dendrogram (C) Gene enrichment analysis of differentially accumulated proteins in PPI responder patients. Representation of the most relevant Gene Ontology (GO) terms related to biological processes. The size of the dot represents the number of proteins from our data set related to each process. Dots are colored according to their significance, which is set by a color scale referring to −log10 (adjusted *p*-value). Left (upregulated in responder-PostPPI), right (downregulated in responder-PostPPI).

It has been described that 39% of the esophagus-specific transcripts (117) are altered in esophageal biopsies from EoE patients (Eso-EoE panel) being around 90% of them downregulated.[38] Using the current data available at the Human Protein Atlas site,[34] we found that 40 differentially accumulated proteins from the PPI-responder comparison were esophagus-enriched genes, of which 38 were upregulated and only 2 downregulated in responder-PostPPI versus responder-PrePPI (Figure S2 and Table S10). Overall, these data suggested that our proteomic analysis agreed with Eso-EoE panel[38] confirming a downregulation of esophagus-enriched genes and a profound loss of proteins related to esophageal epithelial differentiation in EoE.

3.6 | Prediction of PPI response in EoE: Protein signature in PPI responders versus non-responders at baseline

The identification of molecular factors that might predict response to PPI in patients with EoE has been largely elusive.[12] In our cohort, there were no significant differences between responders and non-responder patients at baseline in demographics, symptoms, and endoscopic and histologic findings (Table 1).

To further assess whether protein signatures at baseline differed, protein profiles from esophageal biopsies were compared between responder-PrePPI and non-responder-PrePPI EoE. First, the global transcriptomic profile was analyzed by PCA showing no differences in the transcriptomic signature between responder and non-responder patients across both the first and second principal component, which accounted for 31.95% and 10.06% of total sample variance, respectively (Figure 5A). Our transcriptomic analysis revealed that no differentially expressed genes between groups were detected at baseline (adjusted *p*-value ≤0.05 and fold change>2) (Table S12). These results agreed with previously reported studies that failed to find any molecular difference between these two groups.[12] Interestingly, when this analysis was performed using proteomic data, PCA results showed a clear separation between responder-PrePPI and non-responder-PrePPI patients across the second principal component, which accounted for 7.4% of total sample variance (Figure 5B). Further comparative analysis of protein accumulation between these two cohorts identified 28 proteins (2% of total detected proteins) (adjusted *p*-value ≤0.05 and fold change>1.5) (Figure 5C). Specifically, 12 proteins were upregulated and 16 were downregulated in non-responder-PrePPI when compared to responder-PrePPI (Figure 5; Table S13). In addition, a supervised heatmap was generated using the differentially accumulated proteins from the previous analysis showing a clear difference in the pattern of protein accumulation (Figure 5D).

To identify the over-represented Gene Ontology terms (biological processes) associated with the these altered proteins (with *p*-value ≤0.05 logFC >1.5) between responders and non-responders PrePPI patients, we performed gene enrichment analysis. Interestingly, non-responder-PPI patients presented increased levels of proteins related to regulated exocytosis and antigen presentation processes (Figure 5E).

Finally, to further confirm these observations we validated the expression of two selected proteins by immunofluorescence analyses in representative tissue samples (responder-PrePPI and non-responder-PrePPI EoE patients): HM13 and PPME1 (Figure S3). Finally, according to the Gene Ontology database, 22 proteins were categorized as intracellular, 11 as membrane, and 11 as secreted (Figure S1, Panel B; Table S13). As previously mentioned, secreted proteins could potentially be detected in blood samples or through devices like the String test.[35]

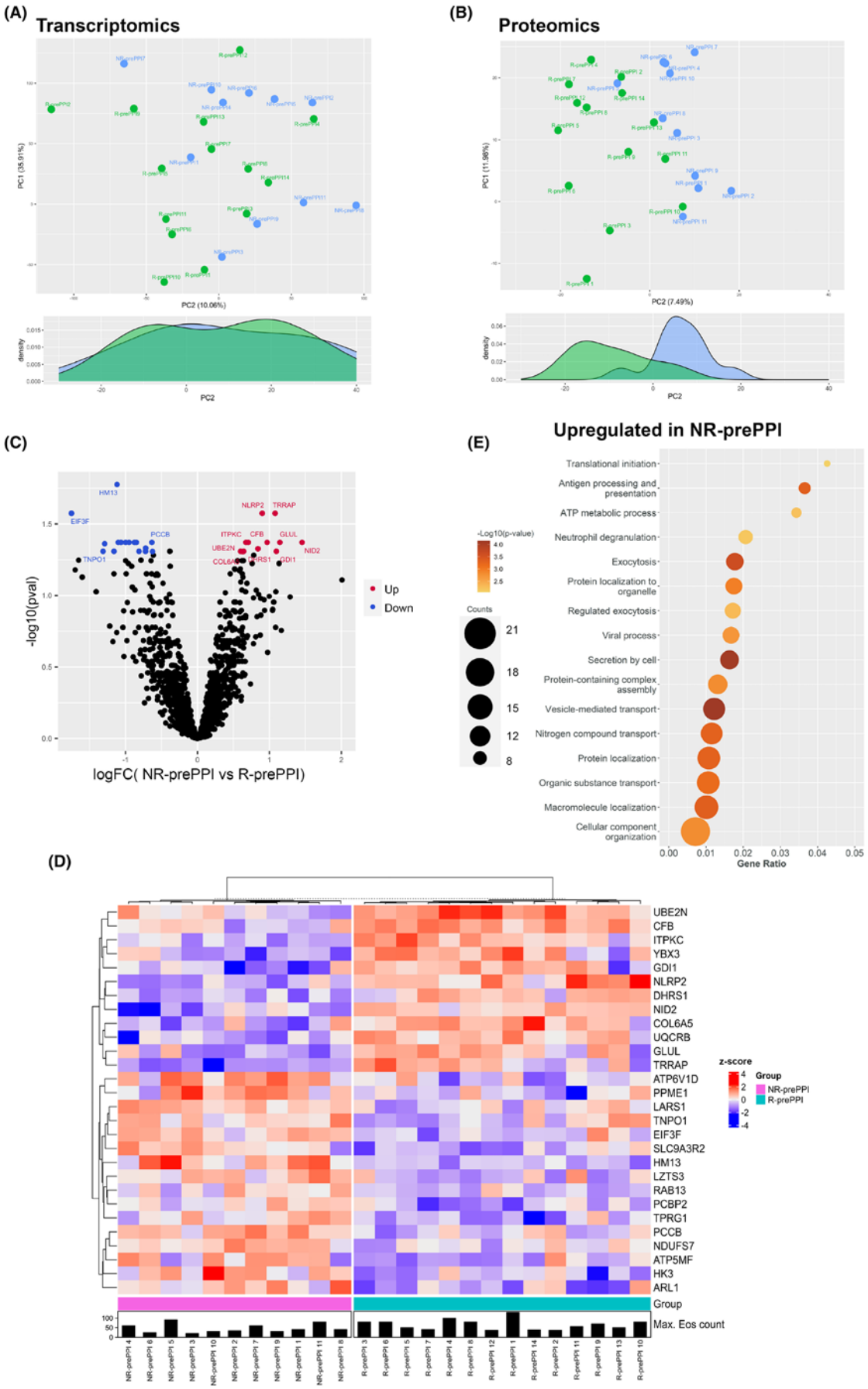
In summary, our analyses showed no substantial differences in baseline mRNA levels between EoE patients who will respond to PPI therapy and those who will not. However, we found a specific protein signature able to explain a percentage of the variability between responding and non-responding patients before PPI treatment.

4 | DISCUSSION

Herein, we used a proteomic approach to provide, for the first time, an expanded view of the molecular changes occurring within the inflamed esophageal mucosa of EoE patients treated with PPI. We found that untreated EoE patients at baseline, including PPI responders and non-responders, have a similar altered protein signature in the esophageal tissue when compared to control individuals (Figure 1), providing compelling evidence that the two groups are part of the same disease.[39] Nonetheless, the resulting differentially accumulated protein panel (Figure 2C), which includes proteins involved in inflammatory process and epithelial differentiation, was almost reverted after PPI treatment in EoE responder patients (Figures 1–4). We confirmed that the EoE esophageal proteome reflects the esophageal tissue alteration, associated with a chronic eosinophilic inflammatory condition, partially reversed by an effective PPI treatment. These results agree with a persistent alteration in the esophageal gene signature observed in EoE patients, even with a deep remission.[40]

PPI response in EoE appears as a continuous varying from null changes in esophageal biopsies to complete normalization of

FIGURE 5 Comparative protein expression in responder and non-responder patients at baseline. (A) Representation of PCA of whole transcriptome data, PC1 on *y* axis and PC2 on *x* axis. Percentage of explained variance is indicated on each axis. The samples are colored by group; responder-Pre-PPI (green), non-responder-PrePPI (blue). (B) PCA representation of proteomic data, PC1 on *y* axis and PC2 on *x* axis. Percentage of explained variance is indicated on each axis. The samples are colored following the same key as in A. (C) Volcano plot representation of the comparative expression analysis in the two Pre-PPI EoE cohorts. Log2 fold change is represented on the *x* axis, and −log10 of adjusted *p*-values on the *y* axis. The proteins are colored by relative expression values: Upregulated or downregulated in non-responder-PrePPI (red and blue, respectively). Comparative expression criteria are adjusted *p*-value ≤0.05 and a 1.5-fold change. Gene names are shown for the most extreme values. (D) Heatmap showing z-score scaled protein expression from 28 differentially expressed proteins between responder-PrePPI and non-responder-PrePPI EoE patients. Sample group is indicated on the bottom bar, with the maximum eosinophil count from each subject. The proteins are hierarchically clustered using Euclidean distance, as shown in the left dendrogram. (E) Gene enrichment analysis of differentially expressed proteins non-responder-PrePPI versus responder-PrePPI. Representation of the most relevant Gene Ontology (GO) terms related to biological processes. The size of the dot represents the number of genes from our data set related to each process. Dots are colored according to their significance, which is set by a color scale referring to −log10 (adjusted *p*-value).



mucosal histology and symptoms recovery. It has been previously reported that some EoE patients have a decline in eosinophil count after PPI treatment without achieving histologic response, whereas in other cases histologic parameters such as lamina propria fibrosis, eosinophil degranulation, and basal cell hyperplasia completely normalize.[29,41,42] This continuous spectrum of changes is simplified into the dichotomous variable "response/non-response" due to its clinical usefulness, depending on whether or not patients reduce the density of esophageal eosinophils below the diagnostic threshold of 15 cells per hpf. Interestingly, although no differentially accumulated proteins were found between non-responder-PrePPI and non-responder-Post PPI (Figure 2; Table S7), a partial recovery of the protein signature was also observed in non-responder patients (Figure 1). More in-depth analysis of the non-responder group revealed that 4 patients (36.3%) achieved partial reduction in the eosinophil count after PPI treatment, with 2 patients (18.2%) having ≥50% decrease. In particular, several non-responder-PostPPI patients showed a clear shift towards the protein profile observed in control or responder-PostPPI patients (Figure 1). In these patients, the histologic index (EoE-HSS) before and after PPI treatment was substantially reduced, regardless of the eosinophil count. These findings raise the question of whether there is a subgroup of EoE patients who may benefit from ongoing PPI treatment, even in the absence of a histologic response.[43] Around 70% of those EoE patients in whom double PPI doses achieve histological and clinical remission maintain long-term remission after a dose reduction.[43,44] Our results demonstrate that short-term PPI treatment restores the accumulation of proteins related to inflammation and epithelial differentiation processes such as cornification (Figure 4), suggesting that it might also prevent long-term complications of EoE such as fibrous remodeling and esophageal narrowing.[42] However, whether PPI maintenance therapy, usually after tapering to the minimum effective dose, preserves this non-inflamed protein signature needs to be determined.

In patients with clinical and histologic features of EoE, genotypic and phenotypic features of PPI responders and non-responders are indistinguishable.[12] Despite its favorable risk/benefit profile,[45] PPI may have short- and long-term adverse side effects making unnecessary or too long exposure risky for patients.[12] Therefore, clinical, symptomatic and/or molecular approaches to identify PPI response before intervention would be an important advance for patient care. In our work, we identified a specific protein profile (Figure 5) capable of predicting PPI response before starting treatment. When we categorized differentially accumulated proteins between R and non-responder PrePPI, they were involved in two main signaling pathways related to regulated exocytosis and antigen presentation (Figure 5). Considering the upregulation of antigen presentation at tissue level (Figure 5), our observations could be indicating a high infiltration of these cells in the esophageal tissue during the sustained inflammation. These patients might present a more altered barrier in the esophagus, increasing the risk of antigen infiltration, thereby favoring EoE worsening, as described before.[10] In the case of swallowed topical corticosteroids, inflammatory mediators have been proposed as predictors of treatment response[46]; however, inappropriate formulas unable to properly adhere to the inner esophageal surface hindered these results. Our results described a protein signature at baseline able to identify non-responder-PPI patients that could help clinicians with decisions on best treatment options. Nevertheless, further analysis is needed to validate whether a protein signature could be used as a predictor for best therapeutic option decisions in routine clinical practice, even for other first-line therapies in EoE such as topical corticosteroids or elimination diets.

Current recommendations for monitoring the therapeutic effect after any treatment in EoE involve serial upper gastrointestinal tract endoscopy with biopsies[4,5,47] This procedure, entails a risk for complications and requires sedation. Identifying non- or minimally invasive biomarkers is not only of high interest for treatment response prediction but also for diagnosis and patient follow-up.[48] Interestingly, since the protein signature is closer to clinical features, our work could lay the groundwork for novel diagnostic biomarkers. Despite our proteomic approach was carried out in esophageal biopsies, according to the Uniprot database, 29 of 102 proteins defined as indicative of patient recovery are classified as secreted proteins (Gene Ontology Term GO:0005576, extracellular-region) (Table S9), while 32 are classified as esophagus-enriched genes. Furthermore, 4 of 28 proteins defined as indicative of PPI response are classified as secreted proteins, and 3 are classified as esophagus-enriched genes (Table S11). Overall, these proteins could be theoretically measured in blood or luminal esophageal samples obtained by minimally invasive methods (as the string test[35] or cytosponge devices[49]).

One strength of this study is the characterization to the first proteomic and RNAseq comparison of esophageal samples between EoE PPI responders and non-responders. These two groups were similar at baseline regarding demographical and clinical variables, and eosinophil counts in esophageal tissue (Table 1). Comparisons with the normal esophagus of control subjects were performed. EoE was exclusively treated with PPI and response objectively assessed in all participants. This study also has limitations: First, the study was conducted at only two referral hospitals for EoE, which could impact generalizability. Second, adherence to PPI therapy was not objectively evaluated. Third, although we were able to provide additional details on biopsy assessment through the evaluation of several histologic features different from eosinophil counts, symptoms were measured with a non-validated tool. Although, the measurement properties of this test have been previously demonstrated,[43,50,51] data must be interpreted with caution. An additional limitation is that PPI treatment in this study was prescribed in a clinical practice setting, and although the doses used followed clinical practice guideline recommendations,[13] no single dosing regimen was used, nor were taken into account clinical variables recently related to a worse response to PPI in EoE, including an stricturing phenotype at endoscopy,[52] low body mass index, high blood eosinophilia,[53] or allergic background.[54] Accordingly, the results of this study may not be applicable to the pediatric population, in which the pathophysiology

of EoE and some pharmacokinetic aspects related to PPI are different from the adult population.[55] Finally, we are aware that biomarker discovery in EoE is hampered since common concurrence of atopic diseases and GERD existed.[4,5] The study of the possible differences in the protein signature of these diseases and EoE would help to understand the common and differential mechanisms of these pathologies and would aid in the validation of the specificity of proteins accumulation as potential biomarkers.

In summary, we have described for the first time that the protein signature of the allergic inflammation associated with active EoE was reverted after PPI treatment in responder patients. Furthermore, despite the protein signature is similar and mRNA signatures are indistinguishable between PPI responding and non-responding patients at baseline, differential protein accumulation between them suggest new potential biomarker predictors, even through non-invasive methods.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: F.M.-J, A.J.L., C.S., P.M. *Participated in the clinical management of patients*: S.C., J.A.M.-M, M.T.F.-P, V.M.-D, J.F.-P, P.M.-H, A.J.L., C.S. *Performed the experiments*: F.M.-J, L.U.-T, L.A.-G, E.A, E.J.L.-M, C.R.-R. *Analyzed and discussed the data*: F.M.-J, L.U.-T, E.A., C.R.-R, J.G.-M, J.M., A.J.L., C.S., P.M. *Wrote the paper*: F.M.-J, L.U.-T, P.M. All the authors read, provided comments, and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

None to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. Additional data set will be available upon request at time on publication. Please contact the corresponding author for any inquiries.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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