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ORIGINAL ARTICLE

Proteomic analysis of the esophageal epithelium reveals key features of eosinophilic esophagitis pathophysiology

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Abstract

Background: Eosinophilic esophagitis (EoE) is a chronic non-IgE-mediated allergic disease of the esophagus. An unbiased proteomics approach was performed to investigate pathophysiological changes in esophageal epithelium. Additionally, an RNAseq-based transcriptomic analysis in paired samples was also carried out.

Methods: Total proteins were purified from esophageal endoscopic biopsies in a cohort of adult EoE patients ($n=25$) and healthy esophagus controls ($n=10$). Differentially accumulated (DA) proteins in EoE patients compared to control tissues

Abbreviations: APOA4, apolipoprotein A4; CRNN, cornulin; DA, differentially accumulated; DCN, decorin; DE, differentially expressed; DSG, desmoglein; DSP, desmoplakin; EDP, EoE diagnostic panel; EoE, eosinophilic esophagitis; FDR, false discovery rate; FLG, filaggrin; GERD, gastroesophageal reflux disease; GO, Gene Ontology (GO) terms; GSEA, gene set enrichment analysis; hpf, high-powered field; IL, interleukin; IPA, Ingenuity Pathway Analysis; IVL, involucrin; KRT, keratin; PCA, principal component analysis; POSTN, periostin; PPI, proton pump inhibitors; RNASE, ribonuclease A family member; RNAseq, RNA sequencing; ROCK2, RhoA-associated coiled-coil containing protein kinase 2; SERPINC1, serpin family C member 1; STAT3, signal transducer and activator of transcription 3; TR, transcriptional regulator; TGFβ1, transforming growth factor beta 1; ZO-1, zona occludens 1.

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were characterized to identify altered biological processes and signaling pathways. Results were also compared with a quantitative proteome dataset of the human esophageal mucosa. Next, results were contrasted with those obtained after RNAseq analysis in paired samples. Finally, we matched up protein expression with two EoE-specific mRNA panels (EDP and Eso-EoE panel).

Results: A total of 1667 proteins were identified, of which 363 were DA in EoE. RNA sequencing in paired samples identified 1993 differentially expressed (DE) genes. Total RNA and protein levels positively correlated, especially in DE mRNA-proteins pairs. Pathway analysis of these proteins in EoE showed alterations in immune and inflammatory responses for the upregulated proteins, and in epithelial differentiation, cornification and keratinization in those downregulated. Interestingly, a set of DA proteins, including eosinophil-related and secreted proteins, were not detected at the mRNA level. Protein expression positively correlated with EDP and Eso-EoE, and corresponded with the most abundant proteins of the human esophageal proteome.

Conclusions: We unraveled for the first time key proteomic features involved in EoE pathogenesis. An integrative analysis of transcriptomic and proteomic datasets provides a deeper insight than transcriptomic alone into understanding complex disease mechanisms.

KEYWORDS
basic mechanisms, eosinophilic esophagitis, epithelium, proteomic

1 | INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic non-IgE-mediated allergic disease of the esophagus. It is clinically characterized by symptoms of chronic or recurrent esophageal dysfunction (e.g., dysphagia, food impaction, heartburn, reflux, chest pain), and histologically by eosinophil infiltration in the esophageal mucosa by over 15 cells per high-power field.[1–5] Other causes of esophageal eosinophilia must be excluded before EoE diagnosis.[1,2] The EoE prevalence exceeds 100 cases per 100,000 inhabitants[6] and it is more frequent in male children and young adults. EoE patients often present other atopies. Currently, EoE constitutes the leading cause of esophageal dysphagia and food impaction in children, adolescents, and adults, and is the second cause of chronic esophagitis after gastroesophageal reflux disease (GERD).[1–5]

Untreated EoE patients tend to exhibit continuous inflammation, which may progress to tissue remodeling, thus leading to rigidity and esophagus narrowing.[7] In most patients, esophageal inflammation recurs after treatment conclusion. Controlling esophageal inflammation in remission is, therefore, key for the long-term management of EoE patients.[8,9] Epithelial injury, with detachment of the external layers, is commonly observed in EoE biopsies, together with tissue regeneration, hyperplasia of the basal layer, and fibrosis in the lamina propria.[10] Notably, esophageal remodeling does not correlate with epithelial eosinophil counts, and inflammatory activity usually dissociates from patients' symptoms.[11,12]

Current EoE anti-inflammatory treatment options include dietary restrictions, use of proton pump inhibitors (PPIs), and topical corticosteroids, which provide variable effectiveness.[13–16] Patients with fibrotic strictures or narrow-caliber esophagus should be assessed with endoscopic dilation. High patient relapse rates and the associated need for long-term therapies have promoted the search of novel EoE treatments, such as corticosteroid-based esophageal-directed formulas[17] and biologics targeting Type 2 immune responses.[18]

The dissociation between clinical symptoms and histology[11,12] and the dependence from endoscopic biopsies for the diagnosis and monitoring of response to treatment represent a challenge in EoE management. In addition, endoscopic biopsies do not assess the full esophageal mucosal surface, nor do they provide complete information of the tissue layers underneath.[10] Consequently, it is of great importance to identify a reliable, non-invasive biomarker that replaces mucosal eosinophilia as a diagnostic and monitoring parameter.[19]

The rapid development of DNA and RNA sequencing technology over the last decade has significantly expanded our vision of EoE.[20] RNAseq recently showed specific patterns of mRNA expression in esophageal biopsies from EoE patients that vary in inflammatory activity[21] and the reverse after topical corticosteroid[22] and PPI[23] treatment. In addition, two significant mRNA EoE-associated signatures have been defined by including esophageal enriched genes: the EoE diagnostic panel (EDP)[24] and the Eso-EoE panel.[25] These EoE-related panels explore molecular mechanisms underlying EoE

pathophysiology, support the existence of EoE endotypes,[26] and describe potential mRNAs as biomarkers of inflammation, fibrosis, and epithelial differentiation.[24,25] In contrast, large-scale proteomic analyses in EoE are still very scarce.[27]

We hypothesized that a proteomic approach with esophageal biopsies from EoE patients could be useful to identify a specific protein signature for this disease. Furthermore, we also considered that a transcriptomic analysis of the same biopsies could ensure reliable data integration regarding EoE physiopathology.

2 | MATERIALS AND METHODS

A detailed description of all the protocols and reagents employed has been included in supplementary materials and methods.

2.1 | Subjects

Adult EoE patients were prospectively recruited at two Spanish hospitals, Hospital Universitario de La Princesa (Madrid), and Hospital de Tomelloso (Ciudad Real) between February 2018 and November 2020. EoE was diagnosed according to evidence-based guidelines[1] as follows: (i) infiltration of esophageal epithelium by 15 or more eosinophil per high-powered field (hpf), (ii) absence of eosinophilic infiltration in biopsy specimens from gastric and duodenal mucosa, and (iii) exclusion of potential causes of esophageal eosinophilia. Controls were subjects who underwent upper endoscopy for assessment of dyspepsia or suspected gastroduodenal ulcer. All selected control subjects exhibited a normal endoscopic appearance of the esophagus, in which hiatus hernia, incompetent cardias, and esophageal peptic lesions were excluded; they did not meet clinical or histological criteria for EoE after endoscopy and biopsy.

For EoE diagnosis purposes, three esophageal biopsies were obtained at the distal and proximal esophagus[1,2] and fixed in 10% buffered formalin before staining with hematoxylin and eosin. Esophageal eosinophilia was defined as an eosinophil count of >15 per hpf (corresponding to an area of 0.24 mm^2) in one or more biopsy specimens. Family and personal history of atopy was recorded in all EoE patients and controls.

The EoE endoscopic reference score (EREFS)[28] rating the severity of esophageal inflammation (edema, furrows, exudate) and fibrostenosis (rings and stricture) was scored in all patients. Furthermore, the validated eosinophilic esophagitis histologic scoring system (EoEHSS)[29] was determined, which evaluates eight pathologic features for both severity (grade) and extent (stage) of abnormalities. One of the EoEHSS features (thickened connective tissue in the lamina propria) was excluded because a large proportion (60%) of samples lacked lamina propria. As previously describe by other authors, nearly half of the esophageal mucosal biopsies obtained in routine clinical practice by using standard forceps had inadequately sampled subepithelium.[29,30]

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

2.2 | Esophageal biopsies processing

Two endoscopic biopsies from proximal and two from distal esophagus were collected in sterile 2-mL cryotubes, immediately snap frozen and stored at −80°C until processing. Proximal and distal biopsies were mixed and processed together. Briefly, under liquid N_2 freezing conditions, biopsies were disrupted using a mortar and pestle, grinding them to a fine powder. The tissue powder was divided in two parts to extract RNA and proteins.

2.3 | Mass spectrometry and bioinformatics analysis

Samples were analyzed in a novel hybrid trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer. A decoy search was conducted to estimate the false discovery rate (FDR) of the searches—only peptides and proteins identified with an FDR < 1% were kept. For the quantitative analysis, only proteins identified with at least two peptides at FDR < 1% were considered for further analysis.

2.4 | Western blot and immunohistofluorescence staining in esophageal biopsies

These assays were carried out using the antibodies listed in Table S1.

2.5 | Pathway enrichment analysis

Differentially accumulated (DA) proteins and genes were analyzed by the overrepresentation test and gene set enrichment analysis (GSEA)[31] using Gene Ontology (GO) terms.

3 | RESULTS

3.1 | Patient characteristics

Clinical and demographic characteristics of study participants are summarized in Table 1. Compared with controls ($n = 10$), EoE patients ($n = 25$) were older (41 vs. 32 years), and more frequently male (88% vs. 60%), but no significant differences were found across groups. EoE patients had more atopic diseases (92% vs. 10%; p-value ≤ 0.001) and exhibited more endoscopic and

TABLE 1 Clinical characteristics of EoE patients and healthy controls.

	Control ($n=10$)	EoE ($n=25$)	p-Value
Sex (male) (n, %)	6 (60%)	22 (88%)	0.155
Age (mean years ± s.d.)	32 ± 11.6	41 ± 13.7	0.088
Symptoms (n, %)			
Dysphagia	0	24 (96%)	<0.001
Food impaction	0	17 (68%)	<0.001
Heartburn	0	8 (32%)	0.07339
Abdominal pain	0	2 (8%)	0.07360
Any atopic disease (n, %)			
Atopic diseases	1 (10%)	23 (92%)	<0.001
Asthma	0	8 (32%)	0.073
Allergic rhinitis/sinusitis	1 (10%)	21 (84%)	<0.001
Food allergy	2 (20%)	7 (28%)	1
Endoscopic findings (n, %)			
Edema	0	21 (84%)	<0.001
Rings	0	13 (52%)	0.03179
Exudates	0	18 (72%)	<0.001
Furrows	0	20 (80%)	<0.001
Stricture	0	5 (20%)	0.2915
Narrowing	0	5 (20%)	0.2915
EREFS (score) (0–9) (mean ± s.d.)	0	4 ± 1.8	<0.001
Maximum eosinophil count (mean ± s.d.)	0	57.3 ± 27.2	<0.001
Histological findings			
EoEHSS grade (0–1) (mean ± s.d.)	0	0.49 ± 0.19	<0.001
Eosinophil density	0	25 (100%)	<0.001
Basal zone hyperplasia	0	20 (80%)	<0.001
Eosinophil abscesses	0	16 (64%)	0.00603
Eosinophil surface layering	0	25 (100%)	0.044
Dilated intercellular spaces	0	24 (96%)	<0.001
Surface epithelial alteration	0	25 (100%)	<0.001
Dyskeratotic epithelial cells	0	7 (28%)	0.193
EoEHSS stage (0–1) (mean ± s.d.)	0	0.44 ± 0.19	<0.001
Eosinophil density	0	24 (96%)	<0.001
Basal zone hyperplasia	0	20 (80%)	<0.001
Eosinophil abscesses	0	16 (64%)	0.001
Eosinophil surface layering	0	12 (48%)	0.017
Dilated intercellular spaces	0	24 (96%)	<0.001
Surface epithelial alteration	0	18 (72%)	0.002
Dyskeratotic epithelial cells	0	7 (28%)	0.084

Note: Fisher's test and Student's t-test were used to analyze significant clinical differences between controls and EoE patients, p-value is indicated.

histological findings associated with the EREFS[28] and EoEHSS scores,[29] respectively. The baseline maximum eosinophil counts were 57.3 ± 27.2 (1–231) in the EoE cases and 0 in the controls (p-value ≤ 0.001).

3.2 | Unbiased proteomic profile in endoscopic esophageal biopsies of EoE patients versus controls

A total of 363 proteins were identified as DA in EoE inflamed samples (using Benjamini–Hochberg corrected p-value ≤0.05 and fold change greater than 1.5; Figure 1A). Specifically, 173 proteins were upregulated, and 190 proteins were downregulated in esophageal samples from EoE patients (Figure 1A and Table S2). Distinct protein expression profiles were identified between control and EoE groups by a principal component analysis (PCA) (Figure 1B). PCA showed a clear separation between control and samples from EoE patients across the first principal component, which accounted for 15.65% of total sample variance (Figure 1A). In addition, a heatmap was generated using the DA proteins from the analysis (Figure 1C). A few EoE samples showed closer distances to control samples (EoE patients 1, 15, 5, 11, and 17), possibly due to lower eosinophil count (Figure 1C).

Next, we compared our results with an esophageal mucosa proteome described in a study containing 32 control human tissues.[32] We found that 85% of our DA proteins were labelled as present in the esophageal tissue (1286 coincidences from 1501 proteins detected in our analysis) (Figure S1).

Finally, we validated the expression level of a group of DA proteins, which are representative of the inflammatory and epithelial alterations observed in EoE, by western blot analysis in four EoE patients and two controls. We selected two proteins that were significantly upregulated in EoE patients (periostin, POSTN, decorin, DCN), two proteins that were significantly downregulated (cornulin, CRNN; involucrin, IVL), and two that were unchanged (zona ocludens 1, ZO-1; desmoplakin, DSP) (Figure 2A). Furthermore, differential accumulation in esophageal biopsies was also confirmed by immunofluorescence analysis (Figure 2B).

To identify biological processes and pathways represented by the DA proteins in EoE patients, we performed a gene enrichment analysis using String software tool[33] (https://string-db.org/). Activation of immune response-related pathways including activation of granulocytes, neutrophils, and leukocytes was significantly enriched in upregulated proteins (p-value $<10^{-11}$, p-value $<10^{-10}$, and p-value $<10^{-9}$, respectively; Figure 3A). Proteins associated with vesicle-mediated transport were also upregulated in EoE tissue, suggesting an active vesicle trafficking in the damaged areas. At the downregulated subset, we identified cornification and keratinization as the most enriched biological processes (p-value $<10^{-9}$ and p-value $<10^{-14}$, respectively; Figure 3B).

Next, protein datasets were analyzed using Ingenuity Pathway Analysis (IPA)[34] (version 60467501) to identify the upstream

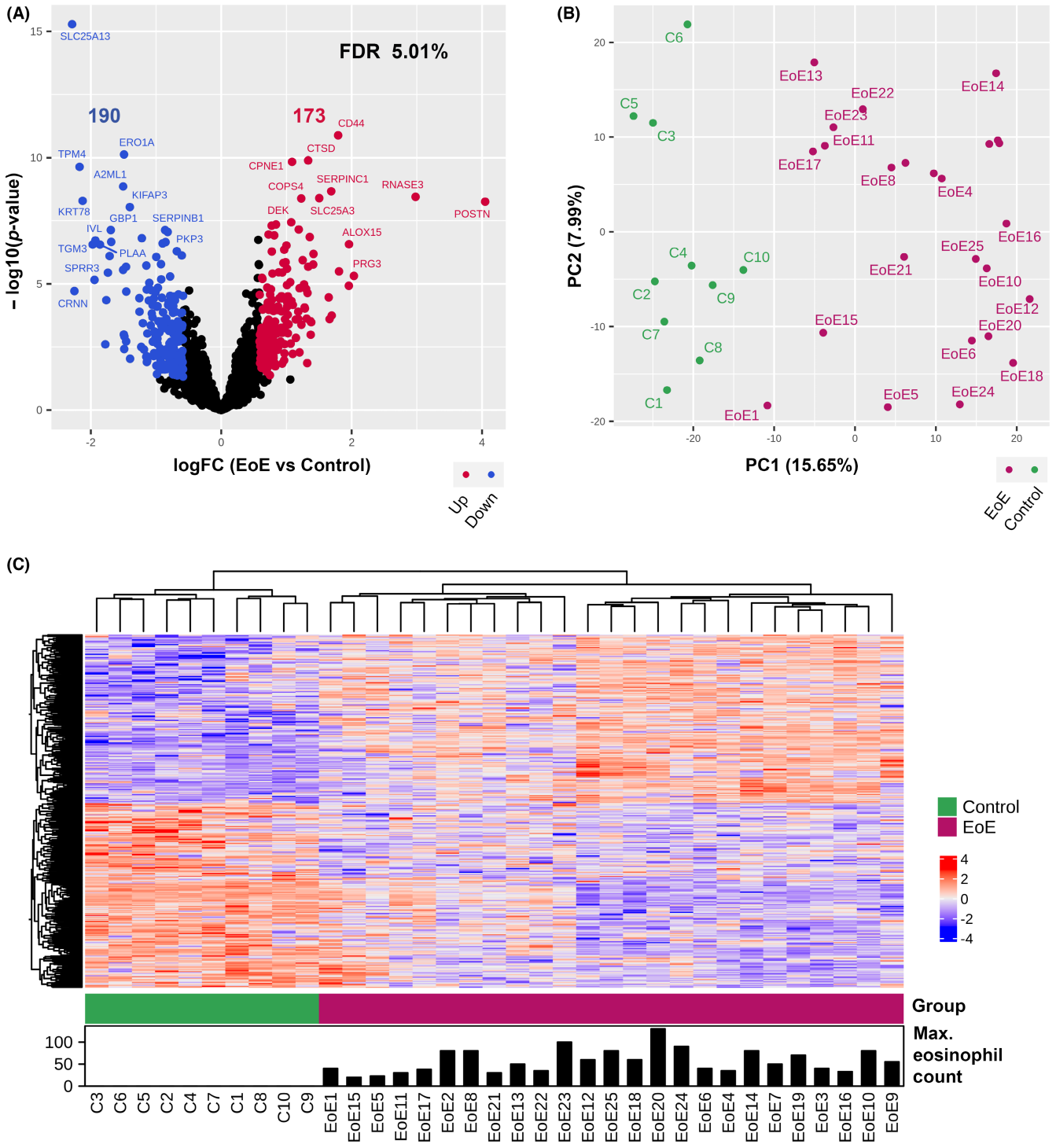


FIGURE 1 Differential protein expression in EoE. (A) Volcano plot representation of the differential expression analysis. Log_2 fold change is represented on the X axis, and −log10 of Benjamini–Hochberg corrected *p*-values on the Y axis. The proteins are colored by relative expression values: upregulated or downregulated in EoE (red and blue, respectively). Differential expression criteria are the adjusted *p*-value of ≤0.05 and a 1.5-fold change. Gene names are shown for the most extreme values. (B) PCA analysis on whole proteomic data. Percentage of explained variance is indicated on each axis. The samples are colored by group: control (green) and EoE patients (purple). (C) Heatmap showing Z-score scaled protein expression from 363 differentially expressed proteins between EoE and controls. Sample group is indicated on the bottom bar, with the maximum eosinophil count from each subject. The samples and proteins are hierarchically clustered using Euclidean distance, as shown in the top and left dendrograms.

transcriptional regulators, and their partners, which may lead to the identification of therapeutic targets for EoE. Each potential transcriptional regulator (TR) presents two statistical measures, a *p*-value (which measures if there is a statistically significant overlap between the dataset genes and the TR-regulated genes) and an activation Z-score (which infers the activation state of predicted

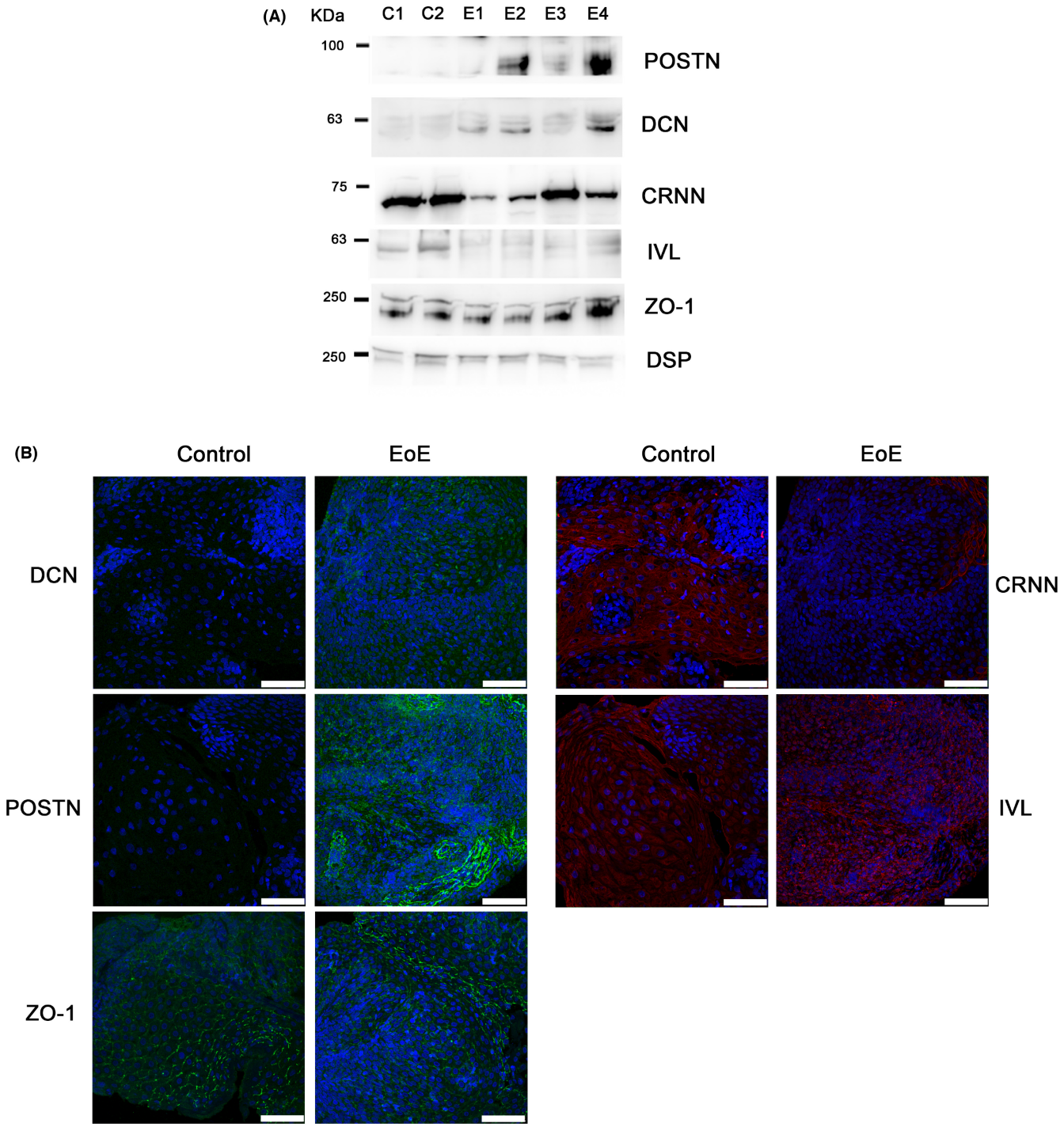


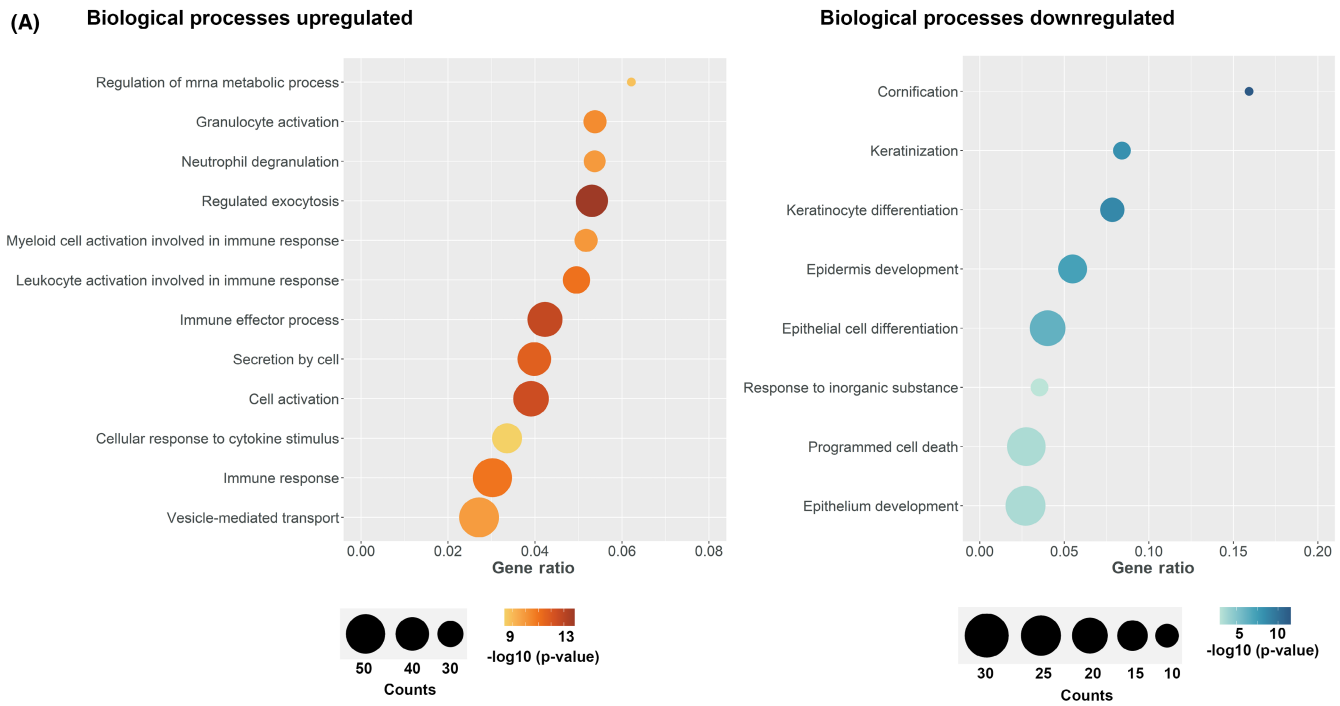
FIGURE 2 Expression of selected dysregulated proteins in EoE. (A) Western blot validation of two upregulated (POSTN and DCN), two downregulated (CRNN, IVL), and two invariable proteins (ZO-1 and DSP) identified by proteomics. Images are representative of two controls and four EoE patients per group. (B) Immunofluorescence analysis of POSTN, DCN, CRNN, IVL, and ZO-1 expression in esophageal biopsy sections from a control and an EoE patient. Tissue sections were stained with pairs of antibodies to simultaneously detect: DCN (green) and CRNN (red), POSTN (green) and IVL (red), and ZO-1 (green). In all images DAPI (blue) is shown. Scale bar 75 μm.

upstream regulators).[34] Figure 3C summarizes the targeted genes by the top upstream regulators (Z-Score >2, Benjamini–Hochberg correction p-value $\leq 10^{-9}$) including two upregulated, transforming growth factor beta 1 (TGF1) and signal transducer and activator of transcription 3 (STAT3). Similar results were obtained using the RNAseq dataset (Figure S4).

3.3 | RNAseq analysis: correlation of mRNA and protein abundance

Given that both transcriptome and proteome data were generated from the same biopsies, we decided to measure the correlation between protein and RNA fold change. We identified a total of 15,674

(A)



(B)

Upstream regulator	Molecule type	Activation state	Activation Z-score	p-value protein	p-value RNA	Molecules
TGFB1	Growth factor	Activated	2.191	<0.001	<0.001	ABCF1,ABL1,ACSL3,ADK,ALB,ALOX15,BGN,CAB39,CALML3,CBR3
STAT3	Transcription regulator	Activated	2.645	<0.001	<0.001	AHSG,ALOX15,APOA4,CD44,CD74,CD9,CFB,COL1A2,CP,CTSB
FOXC1	Transcription regulator	Activated	2.168	<0.001	0.034	CSTA,DSG1,ECM1,GBP1,IVL,KRT1,KRT16,KRT6B,KRT6C,MX1
IL6	Cytokine	Activated	2.677	<0.001	<0.001	AKR1B10,ALB,APOA1,BGN,CD44,CD74,CES1,CP,CRYAB,CTSB

FIGURE 3 Gene enrichment analysis of differentially expressed proteins in EoE. (A) 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). (B) Two main upstream regulators (UR) obtained from the IPA analysis of differentially expressed proteins. For each regulator, the molecule type, activation state, Z-score and adjusted *p*-value are indicated. Genes from our dataset that are implicated in each pathway are presented (Molecules). IPA analysis of differentially expressed genes obtained by RNAseq data. For each regulator adjusted *p*-values are indicated.

transcripts from which 1993 were DE in EoE (adjusted *p*-value ≤0.05, fold change 2.0) (Figure S2). Of these, 851 genes were upregulated and 1142 were downregulated (Figure S2A and Table S3). Accordingly, a clear separation was found between controls and EoE groups by PCA (Figure S2B). This separation was notorious across the first principal component, which accounted for 35.91% of total sample variance. Of note, compared with control samples, EoE tissue samples displayed greater intragroup variation. In addition, a heatmap was generated using the DA proteins from the analysis (Figure S2C).

Next, we compared our RNAseq data with previously reported results obtained with the same experimental approach in EoE esophageal biopsies[21] (GSE58640). When comparing the fold change of all genes between studies, we found a high correlation (Pearson $R = 0.77$, $p\text{-value} = 2.2\ 10^{-16}$; Figure S3).

To identify biological processes represented in DE, we performed gene enrichment analysis of biological processes GO terms. In agreement, activation of immune response, regulation of inflammation, and production and secretion of cytokines were significantly enriched in EoE patients (Figure S4A). However, according to protein data, cornification and epithelial differentiation processes were downregulated at the RNA level (Figure S4B).

Next, we compared fold change from our RNA and protein pairs. Of the 1683 identified proteins, 1550 (92%) were successfully mapped to the RNAseq data (Figure 4A). We calculated a Spearman correlation coefficient of 0.33 between all fold change pairs (Figure 4B). The majority of those RNA–protein pairs (92%) were positively correlated (Figure 4A). In addition, when only common DA protein/mRNA pairs (62) were selected, Spearman correlation coefficient increased to 0.77.

Remarkably, we found 27 proteins in our data for which mRNA levels were undetected in our RNAseq analysis (Figure 5 and Table S4). Applying a selective criterion (fold change >1.5 *p*-value ≤ 0.001) and focusing on the most relevant proteins in EoE, three main groups of DA proteins could be observed (Figure 5A). The first includes Serpin family C member 1 (SERPINC1) and apolipoprotein A4 (APOA4). These are typically synthesized in the liver, where they are enriched at the mRNA level, suggesting a constitutive secretion into blood.[32] A second group includes four eosinophil granule-derived proteins, namely proteoglycan 3 (PRG3), ribonuclease A family member 3 (RNASE3), eosinophil peroxidase (EPX), and RNASE2.[35,36] Furthermore, the levels of these proteins strongly correlate with absolute eosinophil number in esophageal biopsies from EoE patients (Figure 5B) ($0.7 < R < 0.8$). The third group includes two keratins (KRTN-76 and KRTN-86) which have been detected in esophageal mucosa proteome.[32] Interestingly, KRT-86 is also expressed by basophils according to the Human Protein Atlas database. Two upregulated (RNASE3, SERPINC1) and two downregulated proteins (APOA4 and KRT-76) were validated by western blot. Finally, RNASE3 and KRT76 were also analyzed by immunofluorescence in esophageal biopsies.

3.4 | Comparison with two EoE-related mRNA signatures

Next, we compared our protein dataset with the EDP, a quantitative PCR assay of 96 representative EoE genes used as a severity scoring algorithm.[24] In our proteomic data, we detected 18 of them, of which 12 were DA in EoE: 10 upregulated (POSTN, ALOX15, CA2, EPX, COL1A2, TNFAIP6, EPPK1, CFB, CTSC, and APOBEC3A), and 2 downregulated (DSG1 and ENDOU). The majority of these RNA–protein pairs (83.33%) presented the same fold change direction. Intriguingly, two of our DA proteins, EPX and COL1A2, were not described as DE with the EDP[24] (Figure 6A and Table S5).

It has been described that 39% of the esophagus-specific transcripts (117) are altered in esophageal biopsies from EoE patients (Eso-EoE panel). Approximately 90% of them have been described to be downregulated. We next compared our RNA dataset fold change for these 117 transcripts with our protein data and found 31 coincidences between RNA and DA proteins (Figure 6B and Table S6; 30 downregulated and 1 upregulated). The majority of these pairs (96.8%) displayed a similar fold change direction. Since the number of esophagus-specific transcripts has been updated from 2017, we carried out the same analysis using the current data available at the Human Protein Atlas site. We found 56 DA proteins included in esophagus-enriched genes: 50 downregulated and 6 upregulated (Figure 6C and Table S7). Overall, these data strongly suggest that our proteomic analysis agreed with the EoE mRNA-reported panels[24,25] confirming a pattern for esophageal differentiation loss in EoE.

4 | DISCUSSION

Herein, we used a proteomic approach to provide an expanded view of the changes occurring within the inflamed esophageal mucosa of EoE patients. To date, most high-throughput studies focusing on EoE are DNA and RNA sequencing-based.[20] However, measuring both RNA and protein within esophageal biopsies is essential to

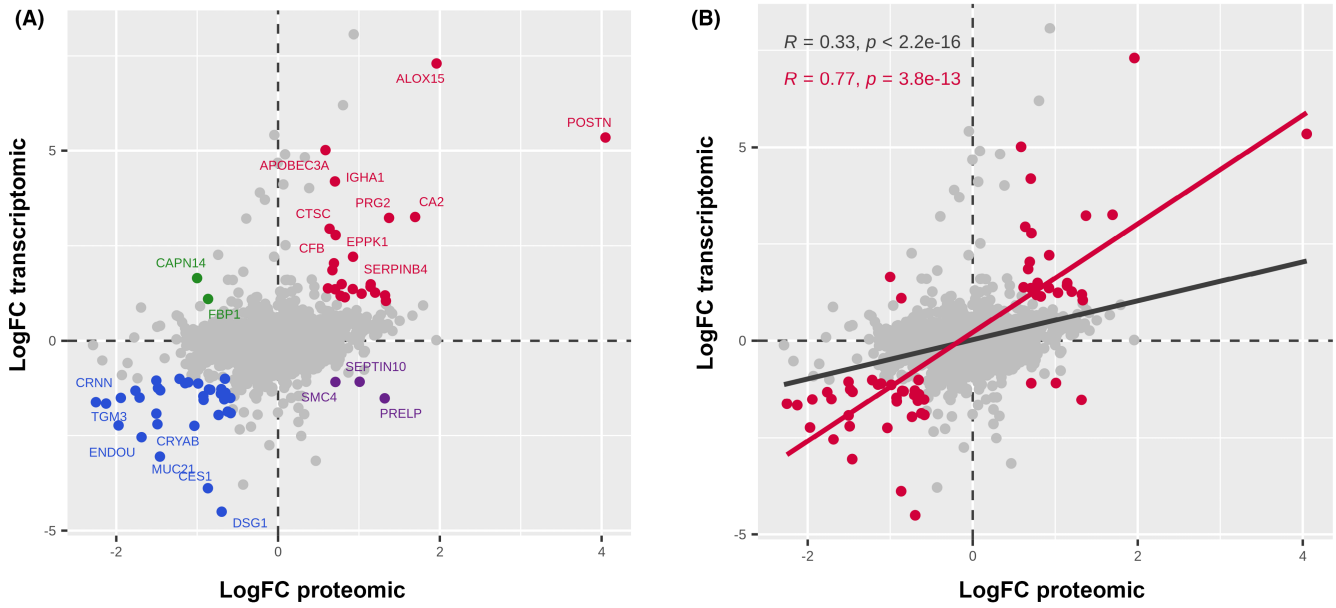


FIGURE 4 Comparative study of proteomics analysis. (A) Comparison of the fold change in proteomics (X axis) and transcriptomics (Y axis). Genes are colored by group (double positive, double negative, and opposite fold change values). Gene names are shown for the most extreme values. (B) Correlation between proteomics and transcriptomics. Pearson's correlation test is shown for all matching data (grey) and for DE genes or proteins in both approaches. R and *p*-values are displayed for each test.

(A)

Gene	Log FC	*p*-value	Description	
RNASE3	2.969	<0.001	Ribonuclease A family member 3	EOSINOPHIL
PRG3	2.035	<0.001	Proteoglycan 3	EOSINOPHIL
RNASE2	1.794	<0.001	Ribonuclease A family member 2	EOSINOPHIL
EPX	1.368	<0.001	Eosinophil peroxidase	EOSINOPHIL
SERPINC1	1.687	<0.001	Serpin family C member 1	LIVER
APOA4	−1.399	0.009	Apolipoprotein A4	LIVER
KRT76	−0.894	<0.001	Keratin 76	MUCOSE
KRT86	−1.489	0.003	Keratin 86	MUCOSE

(B)

	Max. eosinophil count	EoE HSS	EREFS	Body mass
RNASE3	0.84	0.81	0.75	0.18
SERPINC1	0.78	0.81	0.69	0.10
EPX	0.73	0.72	0.56	0.18
PRG3	0.71	0.72	0.60	0.10
RNASE2	0.69	0.74	0.55	0.03
APOA4	−0.21	−0.26	−0.28	−0.19
KRT86	−0.34	−0.42	−0.34	0.13
KRT76	−0.57	−0.66	−0.55	−0.15

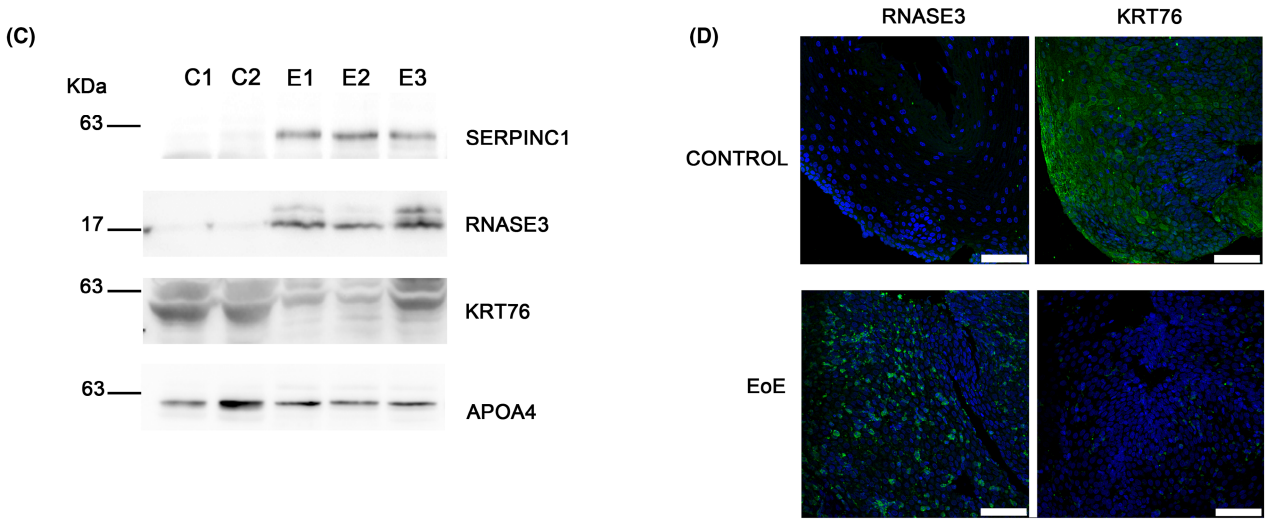


FIGURE 5 Most relevant proteins identified in proteomics but not in transcriptomics in EoE. (A) Gene name, logFC, adjusted *p*-value and a description are included. Background colors are associated with the different tissues/cells expressing these proteins according to the Human Protein Atlas database. (B) Correlation between clinical (EREFS, EOS MAX) and demographical variable (Body mass) and the expression levels of DA proteins in EoE. The Spearman correlation coefficient is represented in red for a positive correlation and in blue for a negative correlation between the outcome scores and inflammatory markers. (C) Western blot validation of DA proteins (SerpinC1, RNase3, KRT76 and ApoA4) found in proteomics but not in our RNAseq analysis. Images are representative of two controls and three EoE patients per group. (D) Immunofluorescence analysis of RNase3 and KRT76 expression in esophageal biopsy sections from control and EoE patients.

fully understand molecular mechanisms underlying EoE, particularly considering that protein abundance often correlates poorly with transcript levels.[37] These discrepancies could be partly attributed to changes during biological processing, such as regulation of translation rate, regulation of protein half-life, and protein transport.[37–39]

The number of proteins detected (Figure 1) was significantly lower compared to transcripts (Figure S1). Yet, our proteomic results further confirm those obtained through mRNA analysis, which are also aligned with previously defined EoE-related gene panels (EDP and Eso-EoE)[24,25] (Figure 6). Immune response-related pathways were significantly enriched in upregulated proteins, whereas cornification and keratinization processes were enriched in the downregulated subset (Figure 3). DA proteins were significantly associated with their mRNA counterpart (Figure 4), although not all proteins were detected at the mRNA level in EoE (6% or 27 proteins). These proteins can be classified in two main groups regarding their tissue of origin (Figure 5). The first group of proteins are synthesized in the liver and constitutively secreted into blood, being enriched in complement activation coagulation, acute phase response, and lipid transport pathways.[32] The presence of these proteins in the esophagus could be explained by the inflammatory process. Inflammation involves the release of a large number of mediators that increase microvessel permeability, cause vasodilatation, and induce leukocyte infiltration. In that inflammatory state, permeability edema is exacerbated, thus resulting in the formation of a protein-rich infiltrate (which could contain these proteins) that increases interstitial fluid volume. In esophageal tissues from EoE patients, an impaired epithelial barrier has been described through histological studies, characterized by dilated interepithelial spaces, basal cell hyperplasia, and a loss of esophageal tissue differentiation.[10,40,41] The second group included eosinophil-related proteins. Eosinophils are naturally present in the gastrointestinal tract under homeostatic conditions, but not in

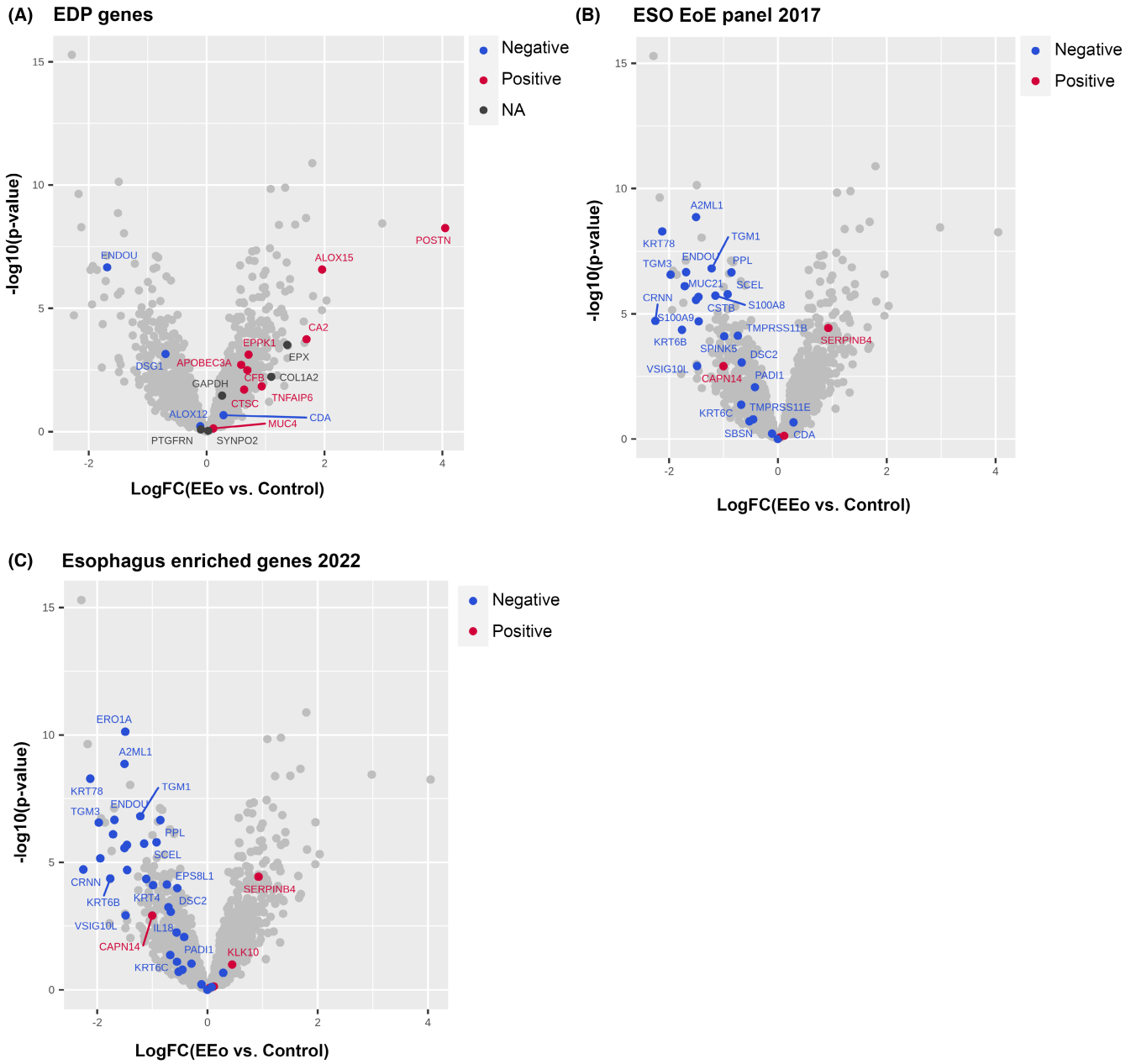


FIGURE 6 Comparative study of proteomics analysis. (A) Volcano plot of protein DA analysis showing the position of the EDP genes.[24] Dot color indicates the value of the fold change in EDP. (B) Volcano plot of protein differential expression analysis showing the position of the Eso-EoE genes panel.[25] Dot color indicates the value of the fold change in transcriptomics assigned to each protein. (C) Volcano plot of protein DA analysis showing the position of the esophagus specific proteome according to Human Protein Atlas 2022. Point color indicates the value of the fold change in our transcriptomics assigned to each protein.

the esophagus. Eosinophil effector functions have been attributed to their capacity to release cationic proteins stored in cytoplasmic granules by degranulation or cytolysis. In agreement with previously reported studies,[35,36] in our RNAseq analysis EPX, RNASE2, and RNASE3 mRNAs were not DE in EoE patients. However, at the protein level, their products were DA. Several results were also confirmed by western blot and/or immunofluorescence (Figure 5). Overall, these data support the presence of mature eosinophils which have previously translated these cationic proteins, stored in cytoplasmic granules. Furthermore, the levels of these proteins strongly correlate with absolute eosinophil number in esophageal biopsies from EoE patients (Figure 5B).

Current recommendations for initial diagnosis and disease monitoring involve serial upper gastrointestinal track endoscopy with biopsies.[1,2] This invasive procedure is expensive, involves a risk for complication, and requires sedation. Identifying non-invasive or minimally invasive biomarkers for diagnosing and monitoring could help medical assistance in EoE patients.[19] According to the Uniprot database, of the 363 differentially expressed proteins determined in our analysis, 87 are classified as potentially secreted proteins (GO Term

GO: 0005576, extracellular region). These proteins could be theoretically measured in blood or esophageal samples obtained by minimally invasive methods (as the cytosponge[42] or string test[43] devices).

IPA upstream regulator analysis identified TRs that could explain the observed DA proteins and DE genes in EoE (Figure 4). Of the top 10 upstream regulators discovered, STAT3 plays a central role in the host response to injury.[44] It is rapidly activated leading to pro-survival programs that assist the host in regaining homeostasis. However, in chronic inflammation it is associated with fibrosis-derived diseases.[44] Although recent evidence suggests that inhibiting STAT3 can prevent kidney fibrosis, its potential role in the control of fibrostenotic events in EoE needs to be determined. Another regulator, TGF-β1 appears to be involved in end-organ dysfunction in EoE and may cause esophageal epithelial mesenchymal transition, tissue remodeling, and fibrosis.[40,45] Inhibition of TGF-β signaling might offer potential for antifibrotic therapies. However, most of the TGF-β1 signaling blockers are associated with unacceptable side effects.

Overall, the DA protein signature defined within EoE patients' biopsies expands our understanding of the pathophysiology of this disease. We confirm that the altered esophageal proteome drives esophageal tissue alteration, which is associated with a chronic eosinophilic inflammatory condition. Our work also suggests that DA proteins substantially extend the mRNA-based EoE molecular signature. This is exemplified by the subset of DA proteins not DE in RNA, which includes liver-secreted and eosinophil-related proteins. Finally, as the protein signature is closer to clinical features, our work could set the ground for novel diagnostic biomarkers, treatment monitoring and therapeutic approaches in EoE.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: FM-J, AJL, CS, PM. Participated in the clinical management of patients: SC, MTF-P, VM-D, JF-P, PM-H, AJL, CS. Performed the experiments: FM-J, LU-T, CR-R, LA-G. Analyzed and discussed the data: FM-J, LU-T, CR-R, EJL-M, AA-A, AC, JM, AJL, CS, PM. Wrote the paper: AJL, CS, PM. All the authors read, provided comments, and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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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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