Oral epithelial dysplasia with lichenoid features shares proteomic overlap with oral epithelial dysplasia without lymphocytic immune response

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Objective. This study investigates the proteomic profiles of oral epithelial dysplasia with lichenoid features (OEDwithLF) and evaluates its relevance as a histopathological feature for lichenoid mucositis (LM) through differential proteomic characterization.
Study Design. SWATH-MS proteomic profiling was conducted on FFPE samples from 6 OEDwithLF, 5 OED cases without associated lymphocytic infiltration, and 5 LM cases. Protein expression levels were quantified and compared. *In silico* analysis examined the biological and molecular functions of dysregulated proteins.
Results. A total of 460 proteins were identified. Unsupervised clustering revealed significant differences between LM and OEDwithLF, with fewer differences observed between OEDwithLF and OED. Bioinformatic analysis indicated dysregulated proteins are involved in nucleic acid binding, ribosome function, and developmental biology. Key potential biomarkers include KRT17, LYSC, CAL5, and CRNN.
Conclusions. The proteomic profile of OEDwithLF is similar to OED without associated lymphocytic infiltration, but significantly different from LM. OED is relevant in lichenoid tissues, and its proteomic changes can be detected. Although OED may coexist with interface mucositis, it is not a defining feature of LM. This challenges the exclusion of epithelial dysplasia from lichenoid diagnoses. Based on this hypothesis-generating study, further investigation is warranted. (Oral Surg Oral Med Oral Pathol Oral Radiol 2025;139:352−363)

Oral lichen planus (OLP) is a chronic inflammatory condition of the mucous membranes, primarily mediated by T-cells. The pathogenesis of OLP involves the activation of cytotoxic CD8+ T-cells, which target and induce apoptosis in the basal cells of the oral epithelium, leading to characteristic histological features of tissue damage.[1] OLP is recognized as an oral potentially malignant disorder (OPMD).[2] The presence of dysplasia within OLP lesions remains a subject of considerable debate, as the histopathological features of OLP can overlap with those of dysplastic changes, leading to diagnostic challenges and potential misinterpretations[3]. This complexity arises due to shared microscopic characteristics, such as basal cell degeneration, band-like lymphocytic infiltration, and epithelial atrophy, which can obscure the identification of dysplastic alterations and increase the risk in under- or overdiagnosing of malignant potential.[4]

Oral epithelial dysplasia (OED) is widely regarded as a precursor to malignancy, playing a crucial role in oral carcinogenesis.[5] The critical question of whether OED should be included as a diagnostic criterion for OLP has generated considerable debate, as it directly

Statement of Clinical Relevance

This study utilizes SWATH-MS proteomics to analyze oral potentially malignant disorders, revealing distinct proteomic profiles for oral epithelial dysplasia with lichenoid features, oral epithelial dysplasia, and lichenoid mucositis, enhancing differential diagnosis and understanding of their malignant potential.

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impacts clinical decision-making. Lesions with dysplasia often exhibit a higher risk of malignant transformation, and distinguishing OLP from OED influences both treatment approaches and monitoring strategies.[6] This diagnostic distinction is essential for determining appropriate interventions and minimizing the risk of progression to mouth neoplasm.[7]

In 2021,[6,7] the World Health Organization (WHO) introduced the term "oral epithelial dysplasia with lichenoid features" (OEDwithLF) to describe tissues that exhibit both lichenoid characteristics and dysplastic changes.[3] This term evolved from the earlier, controversial concept of "oral lichenoid dysplasia," first proposed by Krutchkoff and Eisenberg in 1985.[4] In this context, "lichenoid" is used purely as a histological descriptor, unless otherwise specified. It is essential to clarify that oral lichenoid mucositis (LM) is not synonymous with OLP. As diagnostic criteria for OLP continue to evolve, some researchers have proposed excluding lesions with OED from the diagnosis of OLP, a stance that has fueled debate due to concerns about underestimating the malignant potential of certain lesions.[6]

Given the diagnostic challenges posed by overlapping histopathological features of these disorders, proteomic analysis has emerged as a valuable tool for exploring the molecular mechanisms underlying these lesions and distinguishing between them.[8] One advanced proteomic technology, SWATH-MS, a variant of data-independent acquisition (DIA) methods, allows for precise quantification of multiple proteins within biological samples.[9] This technique complements data-dependent analysis (DDA), which is essential for generating spectral libraries and ensuring qualitative protein identification.[10]

This study aims to compare the proteomic profiles of OEDwithLF, oral leukoplakia (OL) with OED, and non-dysplastic oral LM. Through these comparisons, we aim to determine whether OEDwithLF is a molecularly distinct entity and to assess whether dysplasia should be considered a relevant criterion in the histopathological diagnosis of lichenoid mucositis from a proteomic standpoint.

MATERIAL AND METHODS

Study design and data sets

A nested cross-sectional design, which involves a representative subset of patients, was employed, involving patients histopathologically diagnosed with OEDwithLF, OED without associated lymphocytic infiltration, or LM at a university-based oral medicine clinic in Santiago de Compostela, Spain, between 2010 and 2022. Ethical approval was obtained from the Ethical Review Committee of the Faculty of Medicine and Dentistry at the University of Santiago de Compostela (Ref. 2016/337). This study adhered to STROBE guidelines for reporting observational studies (https://www.strobe-statement.org/checklists/). Written informed consent was obtained from all participants in accordance with ethical guidelines.

Clinical diagnoses of lesions were based on the WHO diagnostic criteria, with consistent standards employed to minimize selection bias.[3] OPMDs with OED were graded using the binary system as either "low-risk" or "high-risk".[11] All patients were followed for over 10 years from their initial OLP/OL diagnosis. Throughout the follow-up period, an exhaustive clinical-pathological correlation was performed by 2 experts in both pathology and oral medicine (P.G.V. and A.B.C.). Patients whose initial OLP diagnosis was unclear based on strict diagnostic/inclusion criteria, or who exhibited features that evolved into other OPMDs such as proliferative verrucous leukoplakia (PVL) or erythroleukoplakia, were excluded. These evolving cases from our unit registry were documented in a previous publication.[12] Patients with a known malignant development during follow-up were also excluded. Additionally, cases with diagnostic uncertainty or insufficient tissue in formalin-fixed paraffin-embedded (FFPE) samples were excluded.

For the generation of a proteomic reference spectral library, 16 FFPE tissue specimens from 16 patients with OLP and OL were used. All cases underwent clinical and histopathological re-evaluation by 2 board-certified oral pathologists (A.I.L.P. and M.P.S.) to confirm the diagnosis before final inclusion. The clinical images were reviewed and approved by C.M.C.P. and I.L.I.M. Clinical photographs, featuring one representative patient from each group, have been included as Figure 1.

Sample preparation, protein extraction and protein digestion

Paraffin was extracted from oral epithelium tissue samples using the method described by Garcia-Vence et al.[13] In summary, 2-3 sections of 10 μm thick FFPE oral epithelium tissue were placed in Eppendorf tubes. Deparaffinization was performed by incubating the sections 3 times in preheated (90-95°C) milli-Q water (400 μL) for 2-3 minutes using a Thermomixer (Eppendorf) set at 500-600 rpm, with a fixed temperature of 95°C. Subsequently, the samples were resuspended in room temperature milli-Q water and stored at −20°C for approximately 12 hours until the protein extraction step.

To extract proteins, the frozen epithelium tissue sections were thawed, and the water was removed. Subsequently, 150 μL of a buffer solution (20 mM Tris-HCl pH 8.8, 2% SDS, 1% CHAPS, 200 mM DTT, 200 mM glycine, and a mixture of protease inhibitors) was added to each sample. The samples were then heated at 100°C for 20 minutes, followed by an additional incubation at 60°C for 2 hours. Next, the samples were

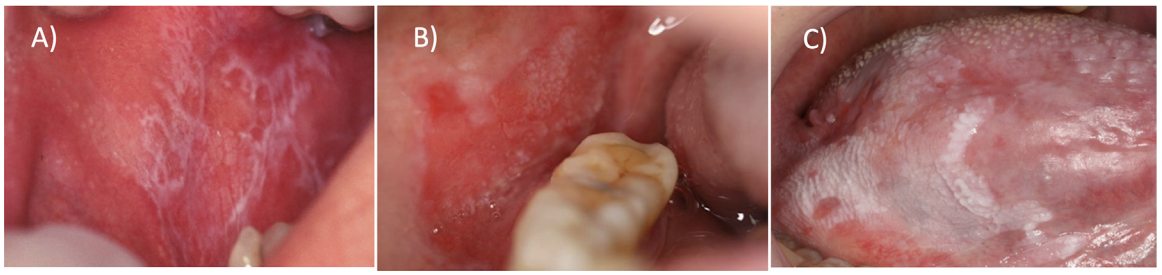

Fig. 1. Representative clinical images for each study group. **(A)** Reticular and erosive lesions affecting the buccal mucosa in a patient with oral lichen planus (OLP) without histopathological presence of oral epithelial dysplasia (OED), included in the lichenoid mucositis group. **(B)** Atrophic/erosive type of OLP on the buccal mucosa histopathologically diagnosed as OED with lichenoid features. **(C)** Non-homogeneous oral leukoplakia on the ventral surface of the tongue, showing high-grade OED on biopsy.

sonicated in a water bath sonicator (Branson Ultrasonics) for 30 minutes. Afterward, the samples were stored at −20°C overnight. The following day, another 50 μL of the buffer was added to each sample, and they were treated with a TissueLyser (Qiagen) for 3 minutes to enhance tissue fragmentation. The samples were then centrifuged at 14,000 rpm for 20 minutes to remove any remaining tissue particles (pellets).[13] The protein concentration in the solution was determined using an RC-DC kit (Biorad Lab.) following the instructions provided by the manufacturer. To remove impurities, 100 μg of protein samples of were subjected to one-dimensional SDS-PAGE. Electrophoresis was carried out until the protein concentrated at the top of the resolving gel (10%) as a single band. This band was then carefully excised using sterile scalpels and cut into small pieces. The pieces were washed with Milli-Q water and 50 mM ammonium bicarbonate in 50% methanol. Dehydration was performed using acetonitrile and a vacuum centrifuge. Subsequently, the protein sample was treated with 10 mM DTT in 50 mM ammonium bicarbonate at 60°C for 30 minutes to reduce it, and then alkylated with 55 mM iodoacetamide solubilized in ammonium bicarbonate at room temperature for 30 minutes in the dark. Next, the protein solution was digested using trypsin (Promega) at a concentration of 20 ng/μL in 20 mM ammonium bicarbonate at 37°C for 16 hours.[14] After that, the extraction of the peptides from the gel was carried out with a solution of 50%(v/v) ACN/0.1%(v/v) TFA (× 3) and ACN (× 1). The resulting solutions were dried and stored at −20°C until further use. To perform the proteomic analysis peptides were resuspended in 0.1 % formic acid.

Generation of the reference spectral library for SWATH-MS analysis

A pool of samples from each group were subjected to analysis using a shotgun data-dependent acquisition proteomic approach by micro-LC-MS/MS. The peptides were separated using the micro-LC system Ekspert nLC425 (Eksigen) with an YCM-TriartC18 column (150 μm × 0.3 mm, 12 nm, s-3 μm) (YMC CO, Japan) at a flow rate of 5 μL/min. A linear gradient of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid) ranging from 5% to 95% B over 30 minutes, followed by 90% B for 5 minutes, and then 5% B for 5 minutes during an equilibration step. The mass spectrometer, a Triple TOF 6600 model (SCIEX), operated in a data-dependent acquisition mode according to the following parameters: 250 ms survey scans from 100 to 1500 m/z (with 25 ms acquisition time), resulting in a total cycle time of 2.8 seconds.[14]

Following MS/MS acquisition, the raw data files were subjected to processing using ProteinPilot 5.0.1 software (Sciex). The Paragon algorithm was utilized for database searching, and Progroup was employed for data clustering. Searches were conducted using a human-specific Uniprot database (Swiss-Prot). The false discovery rate (FDR) was obtained through a non-linear adjustment that correctly identified proteins with an FDR rate < 1%.

Relative quantification by SWATH mass spectrometry

For precise quantification, SWATH-MS was employed using a DIA method. To achieve this, 4 μg of peptides from each individual samples were subjected to liquid chromatography under the same conditions as previously described for the library creation by using a SWATH method. In terms of detection, the mass spectrometer operated with a 50 ms survey scan, followed by MS/MS analysis in a cyclic manner with an acquisition time of 50 ms, resulting in a total cycle time of 6.3 s. Each cycle consisted of 100 scans per SWATH window, with a variable width and a 1 m/z overlap, covering the range of 400 to 1250 m/z.

To process the data, PeakView v.2.2. software (SCIEX) was used for spectral data extraction and

alignment. The extracted data was compared against the reference spectral library mentioned earlier, employing the following settings: a minimum of 10 peptides per protein, 7 fragments per peptide, and an FDR threshold below 1%. Protein quantification was calculated by summing the peak areas from all peptides and fragment ions corresponding to each protein. To extract relevant patterns, principal components analysis (PCA) was applied.

Bioinformatic analysis and interaction networks

Functional analysis was performed by Search Tool for the Retrieval of Interacting Genes/Proteins (STRINGS) software for the analysis of protein-protein interactions (PPIs). Hierarchical clustering of identified proteomic profiles based on relative quantifications by DIA were represented with heat maps by XLSTAT 2.01 (Addinsoft SARL). Individual whisker plots for highly differential expressed proteins among groups as volcanos plots of protein cluster were also produced with GraphPad Prism 8.0 Software. PANTHER tool was also used for of enrichment analysis, particularly focusing on biological processes.

Statistical analysis

For the statistical analysis of the patients' clinical and histopathological attributes, gaussian distribution of the data was determined by the Shapiro-Wilk test along with descriptive measures such as asymmetry and kurtosis. Continuous variables with a normal distribution were reported as mean ± standard deviation, while categorical variables were presented as percentages. In this regard, quantitative variables in multiple independent samples were analyzed using either the student's t-test or the Mann-Whitney U test, and the Kruskal-Wallis test, depending on whether the distributions were Gaussian or non-Gaussian. Statistics were performed using the open-source statistical programming language R (v4.1.2; R Core Team, 2021).

RESULTS

General characteristics of the included cohorts

A total of 16 sequential samples were included in this study, particularly: (a) 6 patients with OEDwithLF, (b) 5 patients with LM, and (c) 5 individuals with OED (Figure 1). Mean age of participants was 64.27 ± 6.24. Most of the cohort was built by women with 12 participants (75%). No differences were found regarding age (p = .68) and sex (p = .24) Only 4 patients were either current smoker or alcohol consumer. Clinical appearance of LM and OEDwithLF is descripted in Table I. In the case of oral white patches, these were clinically diagnosed as OLs, with plaque-type OLP being excluded.[12] In terms of OED grading among the affected groups, there was only 1 case with high-grade dysplasia in the OEDwithLF group, while 2 in the OED subset according to binary grading system. Representative photomicrographs with H&E staining of each subgroup are displayed as Figure 2. General description of the participant variables and their association with the diagnostic categories were detailed in Table I.

Proteomic profile analysis among oral potentially malignant disorders

SWATH analysis allowed the identification of 460 proteins (Figure 3). The information collected in this library, including the retention time, MS, and MSMS of all

Table I. Patient demographics and clinicopathological features of selected cases of lichenoid mucositis (LM) retrieved from oral lichen planus, oral epithelial dysplasia (OED) without associated lymphocytic infiltration from oral leukoplakias, and oral epithelial dysplasia with lichenoid features (OEDwithLF)

	LM (n = 5)	*OED (n = 5)*	*OEDwithLF (n = 6)*
Age	58.2 ± 9.4	68.4 ± 8.08	66.2 ± 6.35
Sex	2 (40%) male; 3 (60%) female	2 (40%) male; 3 (60%) female	6 (100%) female
Clinical presentation	2 (40%) erosive OLP: 2 (40%) reticular OLP; 1 (20%) atrophic OLP	3 (100%) homogeneous oral leukoplakias; 2 non-homogeneous leukoplakias.	3 (50%) reticular OLP; 2 (33.33%) erosive OLP; 1 (16.67%) atrophic OLP.
Histopathological diagnosis	5 (100%) hyperkeratosis with chronic lichenoid mucositis	3 (60%) hyperkeratosis and low-grade dysplasia; 2 (40%) hyperkeratosis and high-grade dysplasia	5 (83.33%) hyperkeratosis and low-grade dysplasia with chronic lichenoid mucositis; 1 (16.67%) hyperkeratosis and high-grade dysplasia with chronic lichenoid mucositis
Location of lesions	3 (60%) buccal mucosa; 1 (20%) gingiva; 1 (20%) lateral border of the tongue	4 (80%) lateral border of the tongue; 1 (20%) gingiva	3 (50%) buccal mucosa; 2 (33.33%) lateral border of the tongue; 1 (16.67%) gingiva
Smoking	4 (80%) no; 1(20%) yes	2 (40%) yes; 1 (20%) no; 1 (20%) past use; 1(20%) N/A	6 (100%) no
Alcohol use	5 (100%) no	2 (40%) no; 3 (60%) N/A	4 (66.67%) no; 2 (33.33%) yes

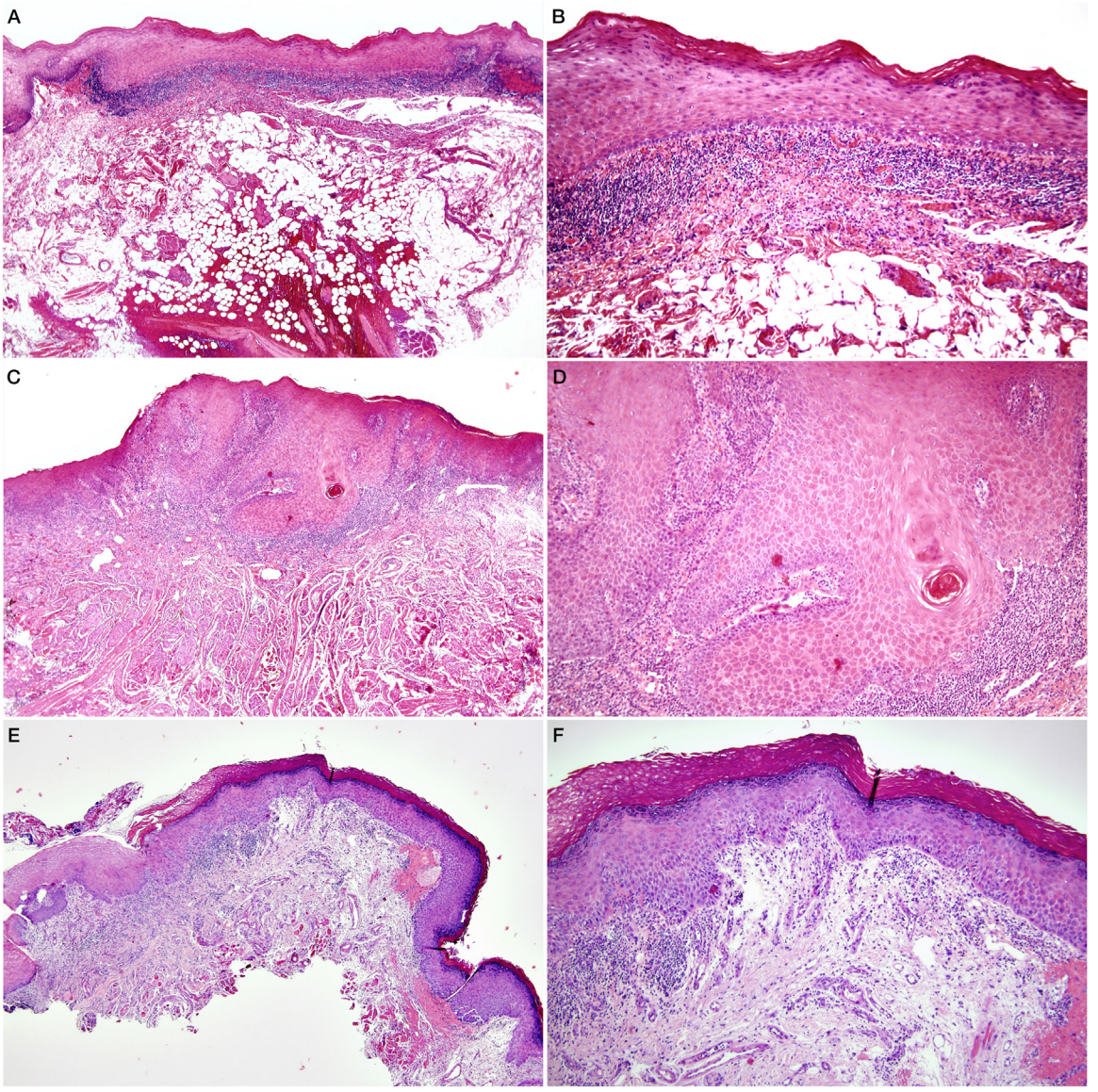

Fig. 2. Photomicrographs with H&E staining of representative cases of 3 study groups. (A and B) Lichenoid mucositis in the context of oral lichen planus (OLP). (C and D) Oral epithelial dysplasia with lichenoid features in the context of clinical OLP. E and F) Oral epithelial dysplasia without lymphocytic immune response in the context of a clinical oral leukoplakia.

peptides in each protein, was used to perform the SWATH analysis. In the PCA, the first 2 principal components (PC) explained 93.3% of the total variation, with PC1 explaining 83.1% and PC2 explaining 10.2% (Figure 3B). Three comparisons were made: (I) LM versus OED, (II) OEDwithLF versus LM, and (III) OEDwithLF versus OED (Figures 3B and 4). In comparison I, 7 differentially expressed proteins ($p < .05$) were found in LM compared to OED. In comparison II, 15 proteins, and in comparison, III, 12 proteins (Table II).

The comparative analysis was represented by a Venn diagram (Figure 3B) of the proteins obtained through the SWATH analysis for the different study groups. The results revealed a total of 460 proteins among the 3 groups. Additionally, 63 common proteins were identified between OED and OEDwithLF, 22 common proteins between OED and LM, and 38 common proteins between LM and OEDwithLF. The findings in the PCA analysis were further validated by means of hierarchical cluster analysis and displayed by heat maps, additionally, noteworthy expression differences among proteins in comparisons were represented with volcano plots (Figure 4).

Protein–protein interaction network and functional pathway enrichment analysis

Molecular functions and biological implications were analyzed to understand the significance of these

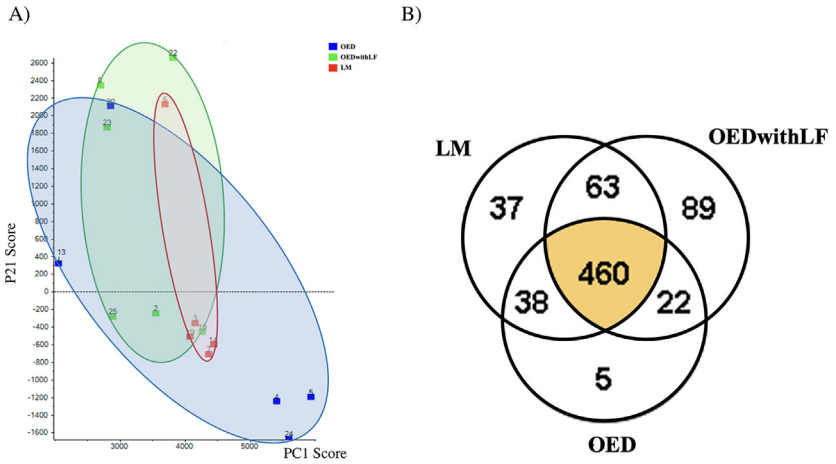

Fig. 3. **(A)** Principal Component Analysis (PCA) in human oral tissue biopsies of different oral potentially malignant disorders (lichenoid mucositis (LM), oral epithelial dysplasia (OED) without associated lymphocytic infiltration, and oral epithelial dysplasia with lichenoid features (OEDwithLF). **(B)** Venn diagram showing the number of unique and overlapping proteins identified in qualitative proteomic analysis of oral potentially malignant disorders subgroups. Abbreviations: PC1 Principal component 1; PC1 Principal component 2.

differential proteins and their possible involvement in the disease's pathophysiology and OED onset. Through STRING analysis (Figure 5), functional enrichment analysis was conducted for the differentially expressed proteins in the various study comparisons.

In the analysis between LM and OEDwithLF, a higher percentage of proteins related to nucleic acid binding, nucleotide binding, ribosomes, and translation initiation were observed (Figure 5A). In this comparison the resulting network was composed of 60 nodes (proteins) and 124 edges (interactions). There were 11 unique proteins this group without interactions with other proteins, but majority of proteins displayed interactions. The *p*-value of PPI enrichment was lower than <1.0e-16 and the local clustering coefficient was 0.527. Regarding biological processes, one cluster was displayed with proteins more strongly linked. The cluster included EIF3CL, RPS27, SYNCRIP, RPS10, SRP14, RPL32, RPL17, TCP1, RPL5, EIF2S1, CCT6A, EIF3B, among others.

In contrast, in the analysis between OED and OEDwithLF, a higher percentage of proteins related to the structure and function of muscle tissue, especially in the context of striated muscle, were observed (Figure 5B). The resulting network was composed of 36 nodes (proteins) and 31 edges (interactions). Additionally, there were 12 proteins without apparent PPIs. The *p*-value of PPI enrichment was 1.65e-06 and the local clustering coefficient was 0.44. Regarding biological processes, 2 clusters were displayed with proteins more strongly linked. One cluster comprised of SPTB, CSRP3, PDLIM3, MYOZ1, MYOM2, MYH6, ATP2A2. The other cluster included PLS3, FSCN1, ATAT1, HLA-A, PTPRC, SERPINB5, THBS1, COL5A1, DSC3, EPPK1, PKP3, FLG, SPRR1B, CASP14. Moreover, these 2 clusters were both connected to developmental biology.

The proteomic profile of the different study groups was analyzed and categorized using PANTHER tool. To clarify the differences in protein composition between the 2 profiles, we performed a global categorization of all the common proteins found between (a) OED and OEDwithLF and (b) LM and OEDwithLF. In both comparisons, common biological processes were observed, such as localization, developmental processes, immune system-related processes, and metabolic processes. In the first comparison, we observed a significant percentage of proteins related to metabolic and cellular processes, biological regulation, and localization. Between LM and OEDwithLF, we also observed proteins related to growth, adhesion, and response to stimulus.

In DIA analysis, certain proteins were predominantly expressed in specific groups of disorders, with minimal or no expression in the others. Notably, LYSC and CRNN were significantly overexpressed in the LM group. Conversely, KRT17 was markedly overexpressed in the OEDwithLF, while CALL5 showed a significant increase in the OED group. The relative quantification of these proteins (KRT17, LYSC, CALL5, CRNN) was performed by normalizing their expression to the overall protein abundance, as illustrated in the whisker plots (Figure 5C).

DISCUSSION

Our findings point to several differentially expressed proteins that may elucidate the molecular underpinnings behind the presence of lymphocytic immune

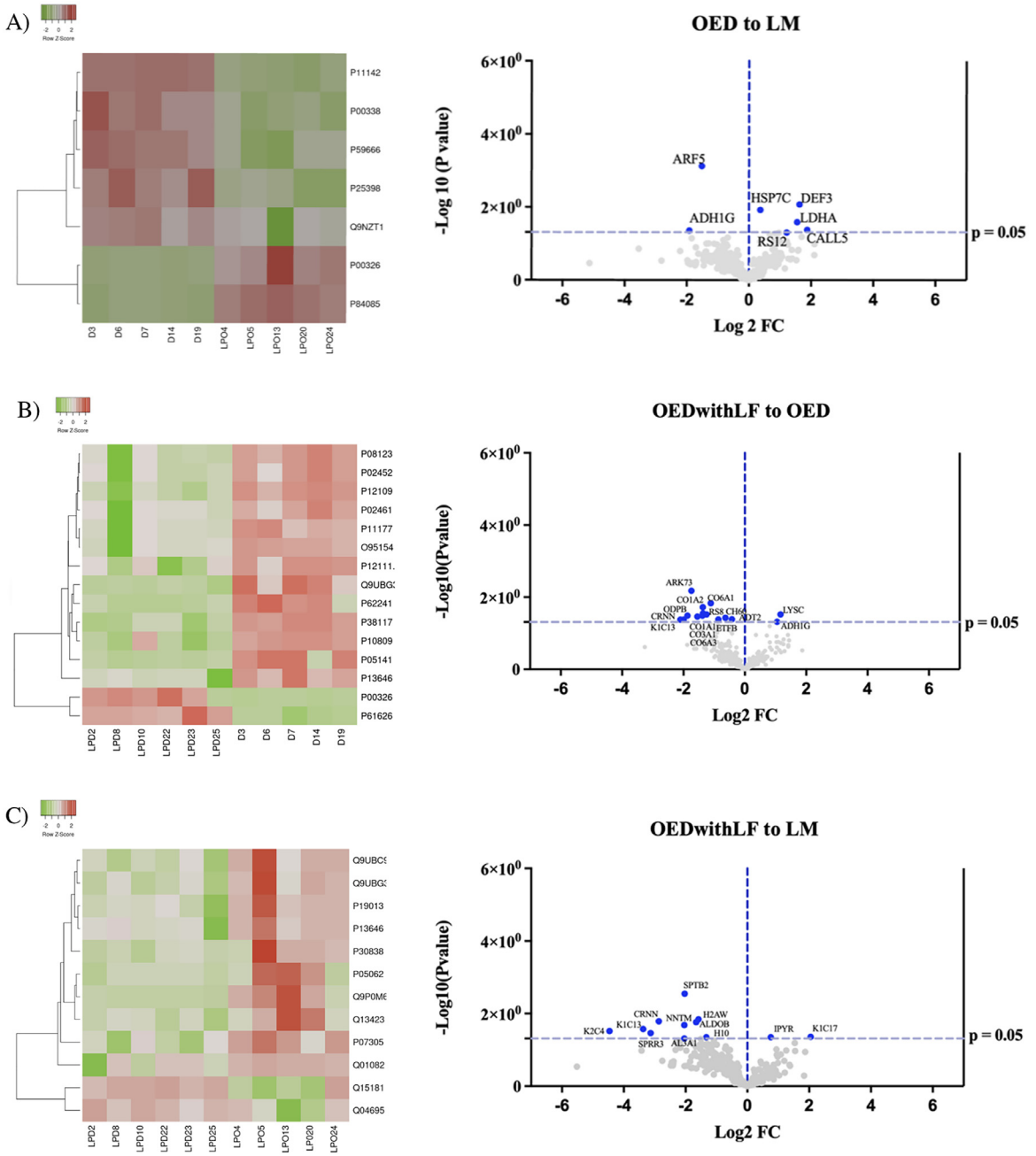

Fig. 4. Representation of dysregulated proteins using a heat map and a volcano plot between different groups. Only proteins expressed significantly differently (≥2 fold and $p < .05$) are shown. In each heat map columns represent each biopsy, and rows represent individual proteins. Protein expression intensities were log-transformed to base 10 and are displayed as colors from red to green. In the Volcano plot graph proteins showing significant expression are located above the dotted line and highlighted in blue. Overexpressed proteins are found on the right side of the graph, while under expressed proteins are located on the left side. (**A**) Oral leukoplakia with epithelial dysplasia (OED) without associated lymphocytic infiltration versus lichenoid mucositis (LM). (**B**) OED without associated lymphocytic infiltration versus oral epithelial dysplasia with lichenoid features (OEDwithLF). (**C**) LM versus OEDwithLF.

response in OED. To the best of our knowledge, this study is the first to employ such an approach, utilizing label-free quantitative proteomics. Overall, our results highlight the close proteomic relationship between the OEDwithLF and OED without associated lymphocytic infiltration groups. In this context, the local tissue microenvironment promoting dysplasia in these potentially malignant oral mucosal disorders seems to harbor a dual precancerous potential, influencing mainly OL but also lichenoid conditions. Moreover, the distilled

Table II. Table with significantly differentially expressed proteins present in the different study groups, namely lichenoid mucositis (LM), oral leukoplakia with epithelial dysplasia (OED) without associated lymphocytic infiltration, and oral epithelial dysplasia with lichenoid features (OEDwithLF)

Accession number	*Protein description*	*Protein symbol*	*Fold change*	*P value*
LM versus OED				
Q9NZT1	CALL5	Calmodulin-like protein 5	↑3.67147217	0.04209727
P59666	DEF3	Neutrophil defensin 3	↑3.09105131	0.00842083
P25398	RS12	40S ribosomal protein S12	↑2.93569729	0.02597155
P00338	LDHA	L-lactate dehydrogenase A chain	↑2.32144942	0.04992188
P11142	HSP7C	Heat shock cognate 71 kDa protein	↑1.28830496	0.01192167
P84085	ARF5	ADP-ribosylation factor 5	↓ 0.3495862	0.00075849
P00326	ADH1G	Alcohol dehydrogenase 1C	↓0.26437046	0.04394936
OEDwithLF versus OED				
P61626	LYSC	Lysozyme C	↑2.23616748	0.02995364
P00326	ADH1G	Alcohol dehydrogenase 1C	↑2.07432141	0.04838922
P05141	ADT2	ADP/ATP translocase 2	↓0.74633286	0.04118929
P10809	CH60	60 kDa heat shock protein, mitochondrial	↓0.64354415	0.03738072
P38117	ETFB	Electron transfer flavoprotein subunit beta	↓0.54923214	0.04157306
P12109	CO6A1	Collagen alpha-1(VI) chain	↓0.46216011	0.01475636
P62241	RS8	40S ribosomal protein S8	↓0.42185334	0.03000387
P02452	CO1A1	Collagen alpha-1(I) chain	↓0.38784874	0.02816793
P08123	CO1A2	Collagen alpha-2(I) chain	↓0.38653382	0.01888723
P12111-4	CO6A3	Isoform 4 of Collagen alpha 3(VI) chain	↓0.38347873	0.03240337
P02461	CO3A1	Collagen alpha-1(III) chain	↓0.34185937	0.03449829
O95154	ARK73	Aflatoxin B1 aldehyde reductase member 3	↓0.29973751	0.00665579
P11177	ODPB	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	↓0.27361952	0.03263667
P13646	K1C13	Keratin, type I cytoskeletal 13	↓0.25060011	0.04208985
Q9UBG3	CRNN	Cornulin	↓0.23320355	0.04169095
LM versus OEDwithLF				
Q04695	K1C17	Keratin, type I cytoskeletal 17	↑4.12849854	0.04412299
Q15181	IPYR	Inorganic pyrophosphatase	↑1.69227691	0.04495249
P07305	H10	Histone H1.0	↓0.39785195	0.04483651
Q9P0M6	H2AW	Core histone macro-H2A.2	↓0.33362575	0.01433688
P05062	ALDOB	Fructose-bisphosphate aldolase B	↓0.31700307	0.01734892
Q01082	SPTB2	Spectrin beta chain, non-erythrocytic 1	↓0.24513964	0.00284372
Q13423	NNTM	NAD(P) transhydrogenase, mitochondrial	↓ 0.2431749	0.02073683
P30838	AL3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	↓0.24294871	0.0489639
Q9UBG3	CRNN	Cornulin	↓0,13685878	0,01632886
Q9UBC9	SPRR3	Small proline-rich protein 3	↓0.11421431	0.03458425
P13646	K1C13	Keratin, type I cytoskeletal 13	↓0.0966017	0.02680421
P19013	K2C4	Keratin, type II cytoskeletal 4	↓0.04528017	0.03048403

Abbreviations: ↑ over expressed; ↓ sub expressed.

proteomic portraits constitute a practical and comprehensive set of proteins that can be considered as candidate disease markers for differential histopathological diagnosis in this triad of oral potentially malignant disorders from a proteomic perspective.

The implementation of a SWATH-MS workflow enabled the identification of differentially expressed proteins among OEDwithLF, LM, and OED. Notably, OED without associated lymphocytic infiltration exhibited a greater number of related proteins with OEDwithLF compared to LM, as confirmed by PCA analysis. Consistent with findings from other authors, the distinctive dysplastic characteristics observed in lichenoid tissues suggest a premalignant feature, indicating that the oral epithelium incurs significant costs from the cytoprotective mechanisms employed to combat autoimmune aggression manifested as lichenoid inflammation.[15] These cellular events may lead to genomic instability and an increased risk of carcinogenesis within the lichenoid spectrum.[16] PCA based on DDA revealed that the LM cluster encompassed both the OED and OEDwithLF clusters. It is important to acknowledge that some residual confounding from physical sampling or individual modifiable and nonmodifiable risk factors may slightly obscure the results, as previously noted by other authors.[9,17]

Secondly, unsupervised hierarchical clustering of OPMD subgroups based on DIA quantification revealed a clear separation, particularly between OED and LM. A closer proteomic profile was observed

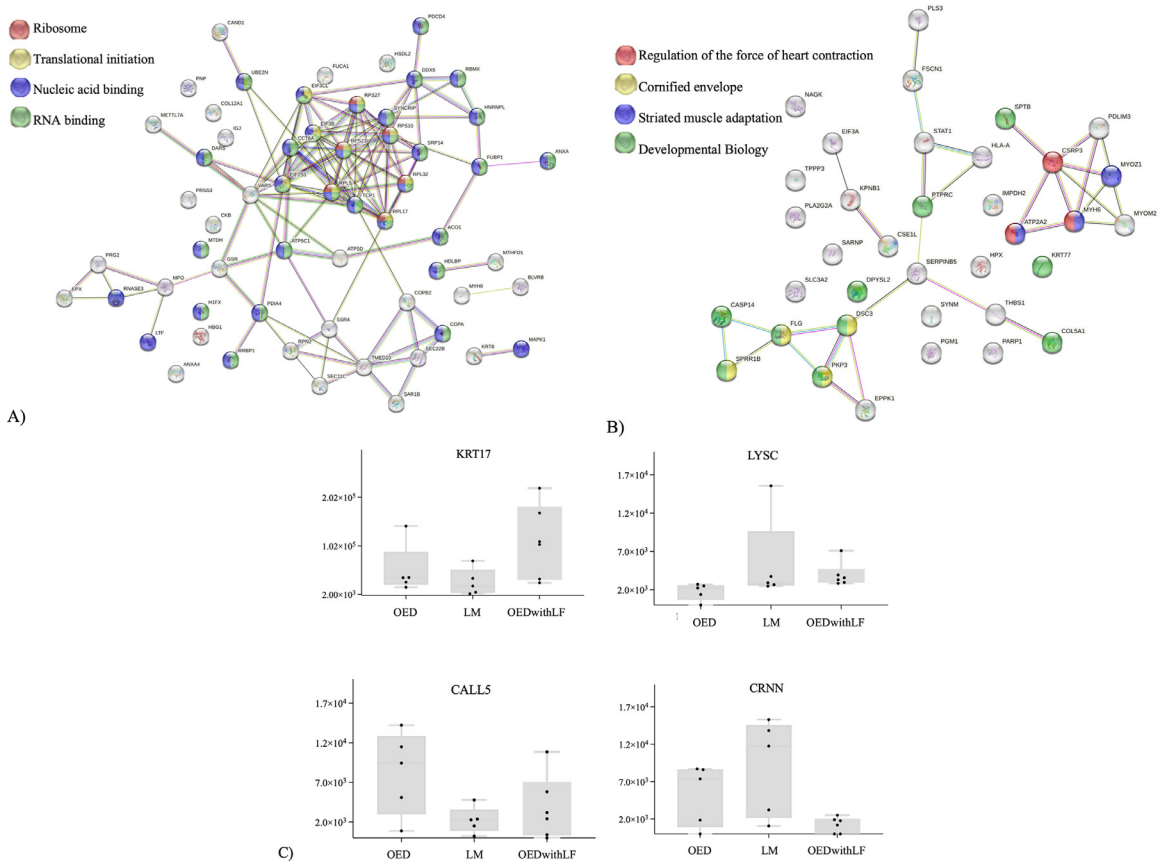

Fig. 5. Functional enrichment analysis conducted with STRING for the differentially and significantly expressed proteins in: (**A**) Lichenoid mucositis (LM) versus oral epithelial dysplasia with lichenoid eatures (OEDwithLF) (**B**) OED without associated lymphocytic infiltration versus OEDwithLF. (**C**) Whisker plot of the percentage change based on DIA quantification. The boxes display the changes in the concentration of KRT17, LYSC, CALL5, and CRNN in each of the 3 groups. The upper error bars indicate the 90th percentile, and the lower error bars represent the 10th percentile, while the central line represents the median.

between OEDwithLF and OED, aligning with our initial hypothesis. These findings contradict a careful genomic and transcriptomic assessment addressing the same pathological challenge carried out by Farah et al.[18] Noteworthy, considering OED as a criterion for excluding a diagnosis of OLP has lately resulted in misclassification of more aggressive forms of OLP, leading to an underestimation of its true potential for malignant transformation.[19,20] In the authors' opinion, accurately determining OED in the context of lichenoid condition is crucial, regardless of clinical or pathological considerations. However, this determination simultaneously presents significant challenges during pathological examination, particularly in terms of interpretation and grading.[20–23] The destruction of basal cells in oral lichenoid tissues leads to secondary changes in lower prickle cells, which subsequently migrate to the basal and suprabasal layers.[24] These activated keratinocytes frequently demonstrate prominent eosinophilic or amphiphilic cytoplasm, irregular cytoplasmic outlines, enlarged eosinophilic nucleoli, and may occasionally exhibit multinucleation.[22] Consequently, lymphocytic infiltration and basal cell destruction can act as confounding factors, potentially masking low-grade OED in the transitional mucositis associated with the lichenoid spectrum.[25] In this context, our perspective aligns with the insightful statement by Lodi et al.,[26] who cautioned against overlooking risk factors for malignant transformation in lichenoid tissues. Disregarding a potential risk factor based solely on the presence of other well-characterized ones is an inadequate approach. Risk factors should not be seen as mere causal agents; ignoring them could not only impede the discovery of new risk factors but also obstruct a comprehensive understanding of those already identified.[27,28]

In addition, we presented a bioinformatic analysis stratified by PPIs and functional enrichment as a proxy for translation dynamics. Specifically, the proteins that exhibited decreased expression in dysplastic OPMDs conveyed consistent biological insights, whereas the proteins with increased expression showed greater

variability. Importantly, PPIs identified by STRINGS in the comparison between LM and OEDwithLF yielded a relevant molecular portrait highly influenced by ribosomes and translation. According to the authors' perspective, this finding may shed light on providing an explanation for the diverse outcomes of this study in comparison to those previously obtained by Farah et al.[18] Farah et al.'s[26] explanation was rooted in results derived from DNA and RNA-based analysis, whereas the current study suggested that translation plays a pivotal role in comprehending the cellular machinery surrounding the emergence of OED in lichenoid tissues. This was corroborated through the analysis of functional enrichment, revealing a network of connections and relationships among these proteins. Supporting evidence from other studies indicated that the distortion of translation may be linked to OED onset in the lichenoid spectrum. In this sense, Xie et al.[29] employed nanoLC-tandem mass spectrometry to profile the proteome in tissues from OLP that eventually underwent malignant transformation, comparing them to tissues that did not exhibit such transformation before the onset of OED. These groups ascertained that malignant development in OLP was linked to CA1, TNNT3, SYNM, and MB overexpression, and FBLN1 underexpression according to DIA-derived data. Also, these networks of proteins were biologically related to actin cytoskeleton, mitochondrial dysfunction, and oxidative phosphorylation pathways, which ultimately linked this malignant transformation to epithelial-mesenchymal transition, as indicated by previous authors.[29] In our study, we encountered a group of proteins also related to the actin cytoskeleton in the OEDwithLF subgroup. It is important to highlight that the study by Xie et al.[29] did not encompass cases of OEDwithLF. Instead, their focus was on biopsies of OLP without malignant transformation, cases with malignant transformation, and oral squamous cell carcinoma (OSCC). Furthermore, the study avoided cases with dysplasia by adhering to the criteria set by van der Meij and van der Waal.[7] This North American research group also utilized a proteomic approach with a diverse pipeline. Consequently, it is understandable that our proteomic analysis did not yield many significant matches.

Furthermore, various proteins have been suggested as potential markers for timely OSCC diagnosis in OLP through various molecular biology approaches.[30] In this vein, a recent scoping review addressing this topic displayed a plethora of molecular changes shared between OLP and OSCC, such as p53 expression, p16/CDKN2A hypermethylation, C-MYC gains, higher Ki67, more apoptosis (BCL-2 and Survivin), and higher MMP-2 and MMP-9 expression.[31] Taken together, these insights corroborate the complex dynamics in lichenoid tissues, suggesting that both immune response mechanisms and protein expression patterns could provide valuable information for improved diagnosis and prognostic assessment in oral mucosal disorders.[32,33]

From a clinical standpoint, OLP and oral lichenoid lesions are the primary OPMD types associated with lichenoid histopathological features.[34] Beyond OLP, which we simplify to LM in our design, there are several related conditions, including oral lichenoid contact lesions, oral lichenoid drug reactions, discoid lupus erythematosus, chronic ulcerative stomatitis, or oral lichenoid lesions related to graft-versus-host disease.[35] Fitzpatrick et al.[36] have highlighted the diagnostic challenge posed by a lichenoid histological appearance, which, according to a large patient audit, is found in over a quarter of biopsies with oral epithelial dysplasia. Recently, the potential for some cases of PVL to evolve from OLP has garnered attention.[3] Published data suggest that this phenomenon might be relatively common.[37–39] Our group has previously reported that PVLs evolving from OLP-like lesions appear to have a lower risk of malignancy compared to those presenting as typical PVLs from the outset.[12] In the present study, we addressed the potential confounding effect of PVL by excluding patients who initially presented with OLP-like lesions and later evolved into PVL. This approach was based on a comprehensive 10-year follow-up, which showed an average transition time of 6.4 years from initial LM histopathological diagnosis to formal PVL in our cohort.[12] Consequently, while some patients may eventually develop PVL or erythroleukoplakia, it is unlikely that those included in our study will transition, given the natural history of the diseases.[40,41]

Our study, however, present with limitations. One key limitation is the relatively small sample size, which, although acceptable for pilot studies, may impact the generalizability of our findings. Additionally, the proteomic analysis conducted did not incorporate a longitudinal approach, which would be instrumental in understanding the dynamics and malignant transformation of lichenoid tissues. Given that this is a hypothesis-generating proteomic study, the characteristics observed may be specific to women and may not fully represent the disease across all sexes. The higher prevalence of lichen planus in women is consistent with our predominantly female cohort, which is appropriate for this scale of study, but results may still be influenced by sex-specific factors. Additionally, buccal mucosal sites were overrepresented in both the LM and OEDwithLF groups, whereas they were inconsistently represented in the OED without associated lymphocytic infiltration group. This limitation arises from the lack of sufficient samples in our institution's tissue registry. It is also important to

highlight the challenge inherent in studying these histological variations within OPMDs, which extends beyond their rarity to encompass the heterogeneity of the samples. Our study protocol did not include OSCC and normal oral mucosa tissue for further comparison. Additionally, it is possible that the signal from less stable proteins may have been reduced because of the use of FFPE tissue despite our exhaustive protocol. Hence, conducting this study on a larger scale with more individuals involved may help overcome these limitations. Furthermore, the regulatory mechanisms governing identified core proteins have not been extensively investigated in cellular and animal experiments.

CONCLUSIONS

This study elucidates the proteomic profiles of OEDwithLF, OED without associated lymphocytic infiltration, and LM. Our findings indicate that OEDwithLF closely resembles OED without lymphocytic infiltration, underscoring the relevance of OED in lichenoid tissues. The presence of OED may signify a premalignant potential in lichenoid tissues. While OED can coexist with interface mucositis, it may also partially define the architectural characteristics of LM, thus challenging the notion of excluding epithelial dysplasia from lichenoid diagnoses. Our research supports the use of proteomic profiling as a valuable tool for differentiating histopathological features in the oral epithelium. Based on this hypothesis-generating study, further investigation is warranted.

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DECLARATION OF INTEREST

All authors have no conflicts of interest to disclose.

DATA AVAILABILITY

The proteomic data and additional histopathological or clinical images from the patients will be made available upon reasonable request to the corresponding author.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Alejandro I. Lorenzo-Pouso: Supervision, Writing – review & editing, Methodology, Conceptualization, Software, Data curation, Formal analysis, Validation, Investigation, Visualization, Resources, Writing – original draft. **Elina Pérez-García:** Writing – original draft, Investigation, Data curation. **Susana B. Bravo:** Investigation, Validation, Formal analysis, Visualization, Resources. **Martina Coppini:** Investigation. **Fábio França-Vieira-E-Silva:** Data curation, Formal analysis, Validation, Investigation. **Cintia M. Chamorro-Petronacci:** Investigation, Formal analysis, Data curation. **Vito Carlo Alberto Caponio:** Supervision, Writing – review & editing, Writing – original draft, Conceptualization, Methodology, Software, Data curation, Resources, Formal analysis, Visualization, Validation, Investigation. **María Elena Padín-Iruegas:** Investigation, Validation. **Irene Lafuente-Ibañez-de-Mendoza:** Writing – review & editing, Resources. **Pilar Gándara-Vila:** Writing – original draft, Supervision. **Mario Pérez-Sayáns:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andrés Blanco-Carrión:** Writing – review & editing, Supervision, Conceptualization.

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