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Histological characterization of the human masticatory oral mucosa. A histochemical and immunohistochemical study

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Abstract

Background: Histology of human oral mucosa is closely related with its function and anatomical location, and a proper characterization of the human masticatory oral mucosa could be very useful in periodontal pathology.

Objective: In the present work, we have carried out a comprehensive study in order to determine the main histological features of parakeratinized (POM) and orthokeratinized (OOM) masticatory human oral mucosa using light and electron microscopy.

Methods: To perform this, we have used several histological, histochemical and immunohistochemical methods to detect key markets at the epithelial, basement membrane and connective tissue levels.

Results: Our results demonstrated that POM and OOM share many histological similarities, as expected. However, important differences were observed at the epithelial layer of POM, that was significantly thicker than the epithelial layer found in OOM, especially due to a higher number of cells at the stratum spinosum. The expression pattern of CK10 and filaggrin revealed intense signal expression in OOM as compared to POM. Collagen and proteoglycans were more abundant in OOM stroma than in POM. No differences were found for blood vessels and basement membrane.

Conclusion: These results may contribute to a better understanding of the pathological conditions affecting the human masticatory oral mucosa. In addition, these findings could be useful for the generation of different types of oral mucosa by tissue engineering techniques.

Research highlights:

- Microscopical features of parakeratinized and orthokeratinized masticatory human oral mucosa showed important differences at both, epithelial and stromal levels.
- Parakeratinized masticatory human oral mucosa exert thicker epithelial layer, especially, at the stratum spinosum in comparison to orthokeratinized human oral mucosa.
- Cytokeratin 10 and filaggrin human epithelial markers were intensively expressed in orthokeratinized masticatory human oral mucosa in comparison to parakeratinized masticatory human oral mucosa.
- At the stromal level, orthokeratinized masticatory human oral mucosa exhibit higher levels of collagen and proteoglycans than parakeratinized masticatory oral mucosa.
- The deep knowledge of histological features of masticatory oral mucosa could lead to a better understanding of oral mucosa pathology and advanced treatments.

KEYWORDS
characterization, histology, human oral mucosa, microscopy, orthokeratinization, parakeratinization

1 | INTRODUCTION

The human oral cavity is subjected to continuous mechanical, chemical and microbiological stimuli (Fleisch & Austin, 1978). Histologically, the human oral mucosa is composed by a stratified epithelium and an underlying stroma (or lamina propria), with both layers being synergically connected by a basement membrane (Ciano & Beatty, 2015; Gómez de Ferraris et al., 2002). The histological and functional characteristics of the oral mucosa show a remarkable degree of variations attending to specific anatomical areas in the oral cavity.

In this milieu, the oral mucosa found in masticatory areas subjected to strong biomechanical forces, such as the gingiva and hard palate, show specific differentiation features, whereas the oral mucosa found at other areas with lower biomechanical requirements is devoid of these features (Sloan et al., 1991). One of the histological characteristics of masticatory oral mucosa that differs among areas of the oral cavity is keratinization, and it has been demonstrated that a close relationship between histology and function exists at this level (Adams, 1976). In this regard, areas of the oral cavity subjected to strong biomechanical forces are usually protected by orthokeratinized oral mucosa (OOM) showing a well-defined cornified superficial stratum (Valach et al., 2017). However, other masticatory regions with lower biomechanical requirements tend to show a parakeratinized epithelial pattern that is characterized by the presence of a noncornified epithelium with flattened cells in the superficial stratum, forming a parakeratinized oral mucosa (POM) (Adams, 1976). Although it is likely that the microscopic differences between both types of oral mucosa may be much more profound and affect all layers of this structure, the number of studies describing the phenotype and histological characteristics of both types of masticatory oral mucosa is very limited, and the histofunctional differences between POM and OOM are not well understood.

In the present work, we adopted a multidisciplinary approach to comprehensively characterize the human masticatory oral mucosa. We employed an array of microscopy research techniques that combine histochemical and immunohistochemical light microscopy with scanning electron microscopy (SEM) to determine the main histological features of POM and OOM at the epithelial, basement membrane and connective tissue levels. This combinatorial approach allowed us not only to evaluate tissue morphology and histology at different levels, but also to analyze tissue composition and histophysiology. These results could contribute to a better understanding of the physiology and pathology affecting both types of masticatory oral mucosa.

2 | MATERIALS AND METHODS

2.1 | Masticatory human oral mucosa samples

Human parakeratinized (POM) and orthokeratinized oral mucosa (OOM) biopsies were obtained from 20 healthy donors undergoing minor dental surgery using local anesthesia. Biopsies were extracted from the gingiva and hard palate areas of the oral cavity and are classified as masticatory mucosa. Average size of the biopsies was 5×5 mm in diameter. Samples were washed in PBS (phosphate-buffered saline) and immediately transferred to the laboratory.

This study was approved by the Institutional Research and Ethics Committee in Biomedical Research of Andalusia (*Comité Coordinador de Ética de Investigación Biomédica*) ref. 0116-N-19, date of approval May, 29th, 2019 and ref. 2044-N-22, date of approval February, 13th, 2023. Informed consent was obtained from all subjects involved in the study.

2.2 | Histological analysis

Samples were divided in two fragments. For light microscopy analysis, one of the fragments was fixed in 4% wt/vol neutral buffered

formaldehyde (Panreac Química S.L.U., Barcelona, Spain). For SEM, the other fragment was fixed in 2.5% glutaraldehyde. Samples fixed in formaldehyde were then dehydrated and embedded in paraffin following routine histology protocols, and 3 µm tissue sections were obtained for hematoxylin and eosin staining (HE) (Panreac Química S.L.U.). Images of the HE-stained samples were obtained using a PANNORAMIC® Flash DESK DW histological scanner (3DHISTECH, Hungary). These images were used to confirm the parakeratinized or orthokeratinized nature of each oral mucosa sample.

For SEM analysis, glutaraldehyde-fixed samples were dehydrated with increasing concentrations of acetone (30, 50, 70, 90, 95, and 100%), dried by using the critical point method, and sputter-coated with gold, according to routine procedures (Campos et al., 2018; Vela-Romera et al., 2019). Specimens were analyzed with an FEI Quanta 200 environmental scanning electron microscope (FEI Europe, Eindhoven, The Netherlands).

2.3 | Histochemical analysis

Several key components of the oral mucosa stroma extracellular matrix (ECM) and the basement membrane were identified by histochemistry. To identify the fibrillar components of the ECM, samples were stained with picrosirius red-PS-(for collagen fibers), Verhoeff staining (for elastic fibers) and Gomori's reticulin metal reduction technique (for reticular fibers) (Sánchez-Porras et al., 2021; Vela-Romera et al., 2019). In brief, for PS staining, samples were incubated in sirius red F3B for 30 min and counterstained with Harris's Hematoxylin for 5 min. Verhoeff staining was performed by incubating deparaffinized tissues in Verhoeff's staining solution for 10 min and further differentiation in 2% ferric chloride for 15 s. And Gomori's reticulin metal reduction technique was performed incubating samples in 1% potassium permanganate, followed by 2% sodium metabisulphite solution and sensibilization with 2% iron alum. After that, samples were incubated in ammoniacal silver and in 20% formaldehyde and the differentiation was performed with 2% gold chloride and 2% thiosulphate.

The nonfibrillar ECM components were evaluated with alcian blue staining-AB-at pH 2.5 (for proteoglycans) and periodic acid–Schiff staining-PAS-(for carbohydrates and glycoproteins). Briefly, for the AB analysis, samples were incubated in alcian blue working solution for 30 min and counterstained with nuclear fast red for 1 min. PAS was performed by incubating each tissue in 0.5% periodic acid solution for 5 min, followed by incubation in Schiff reagent for 15 min and counterstaining with Harris's hematoxylin for 1 min.

2.4 | Immunohistochemical analysis

Specific proteins of the epithelial, stroma and basement membrane layers were detected by indirect immunohistochemistry techniques using the primary antibodies and conditions shown in Supplementary Table 1. First, specific epithelial proteins were analyzed, including several cytokeratins (AE1/AE3, CK5, CK8, CK10, CK13, CK14, CK17), filaggrin (FLG), involucrin (INV), desmoplakin (DSP), claudin (CLDN) and Ki67. Then, the stromal ECM proteins versican (VSC) and decorin (DC) were assessed. To evaluate the vascular network of each type of sample, we carried out an immunohistochemical analysis using the endothelial-related marker CD34 (for small blood vessels) and the smooth muscle actin marker SMA (for large vessels). The facilitate the identification of stromal cells and determine the density and distribution of these cells in the stroma, we used the vimentin marker (VIM). To specifically stain the basement membrane, immunohistochemical analyzes were carried out using anti-collagen type IV (COLIV) primary antibodies.

2.5 | Quantitative and statistical analysis

Quantitative analyzes were performed using ImageJ software (version 1.53 k, National Institute of Health, Bethesda, MD) and following standardized protocols previously described (Carriel et al., 2014; Ortiz-Arrabal et al., 2021; Rodriguez-Pozo et al., 2020; Ruiz-López et al., 2022; Chato-Astrain et al., 2023; Ortiz-Arrabal et al., 2023). Briefly, the histomorphological features of the POM and OOM was assessed measuring epithelial length, thickness, and cell area, as well as rete ridge/papillae dimensions. Then the immunohistochemical analysis were used to quantify signal intensity of specific markers in the epithelium, employing a predefined semiquantitative scale, and in the stroma by mean intensity pixel units.

For statistical analysis, the quantitative results between POM and OOM were compared using nonparametric tests, and a Bonferroni-adjusted p-value of 0.001 was considered statistically significant. Further details and specific values can be found in the supplementary information section.

3 | RESULTS

3.1 | Histological and histomorphological analysis of the epithelial layer of the human oral mucosa

Histological evaluation of the epithelial layer of POM and OOM stained with hematoxylin and eosin revealed the presence of a well-developed epithelium with basal, spinosum, granulosum, and superficial strata in all samples (Figure 1a). However, OOM showed a well-developed granulosum stratum characterized by the presence of keratohyalin granules, and the superficial stratum consisted of abundant flat squamous cells without nuclei showing clear signs of cornification and desquamation, whereas POM was devoid of a granulosum stratum, and the superficial stratum was formed by compact cells containing flat, elongated nuclei (Figure 1a).

When the histomorphological characteristics of the epithelial layer were quantified, we found that the epithelium of POM was significantly thicker than the epithelial layer found in OOM ($p = 0.0008$). However, nonsignificant differences were found for the length and width of the rete-ridges of both types of tissues ($p > .05$) (Figure 1b;

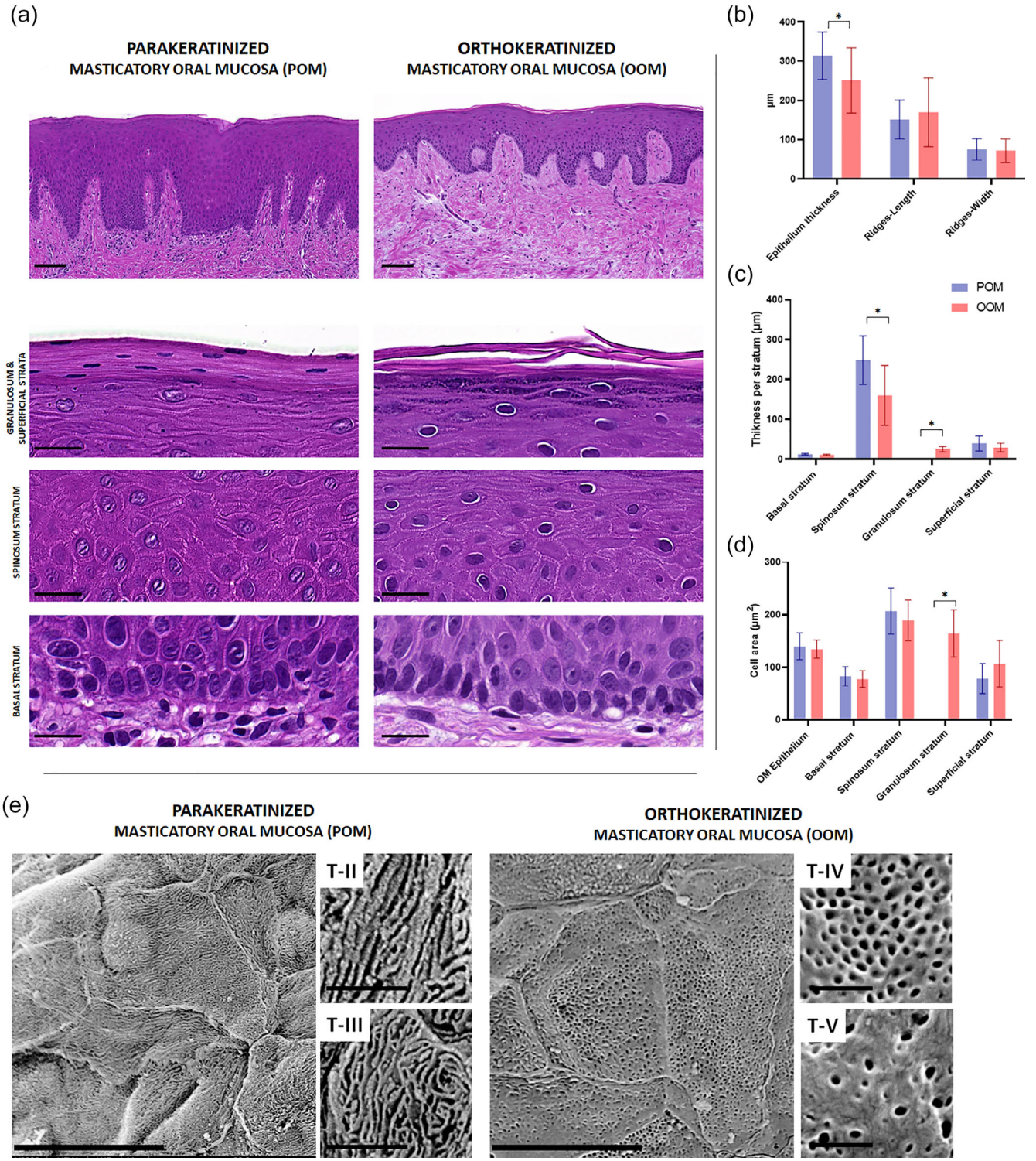

FIGURE 1 Histological analysis of parakeratinized (POM) and orthokeratinized human oral mucosa (OOM) samples. (a) Histological characterization of samples stained with hematoxylin and eosin using light microscopy. (b) Histogram corresponding to the results of the quantitative analysis of epithelium thickness and length and width of the rete ridges. (c) Results of the quantitative analysis of thickness of the basal, spinosum, granulosum, and superficial strata. (d) Results of the quantification of the cell area in the epithelium and in each stratum. (e) SEM analysis of the surface of POM and OOM. Illustrative surface pattern types II, III, IV, and V are shown (T-II, T-III, T-IV, and T-V). Scale bar: light microscopy images 100 µm, SEM images 20 µm and 2.5 µm for inserts. Statistically significant differences were labeled with asterisks in the histograms (*).

TABLE 1 Semiquantitative immunohistochemical analysis of relevant epithelial markers in each stratum of the epithelial layer of parakeratinized (POM) and orthokeratinized (OOM) masticatory human oral mucosa

	Parakeratinized oral mucosa (POM)			Orthokeratinized oral mucosa (OOM)			
	Basal stratum	Spinosum stratum	Superficial stratum	Basal stratum	Spinosum stratum	Granulosum stratum	Superficial stratum
Cytokeratin AE1/AE3	+++	+++	+++	+++	+++	+++	+
Cytokeratin 8	−	−	−	−	−	−	−
Cytokeratin 5	+++	++	++	+++	++	+	+
Cytokeratin 14	+++	++	++	+++	++	+	+
Cytokeratin 10	−	++	++	−	+++	+++	−
Cytokeratin 13	−	++/+++	−	−	++/+++	++/+++	−
Cytokeratin 17	−	++/+++	−	−	++/+++	++/+++	−
Involucrin	−	+++	+	−	+++	+++	+
Filaggrin	−	+/−	−	−	−	++	−
Desmoplakin	+	++	−	+	++	+	−
Claudin	+	++	−	+	++	+	−

Note: Expression at the basal, spinosum, granulosum, and superficial strata of the epithelium. The signal intensity was semiquantitatively determined as strongly positive (+++), positive (++), slightly positive (+) or negative (−).

Supplementary Table S2). Moreover, when the thickness of each epithelial stratum was specifically analyzed (Figure 1c), we found significant differences between both types of samples for the spinosum stratum, with higher thickness in POM ($p < .0001$), and for the granulosum stratum, which was present only in OOM ($p < .0001$). Nonsignificant differences were found for the thickness of the basal and superficial strata ($p > .05$). Regarding the area of the cells corresponding to each stratum, differences between POM and OOM were statistically significant only for the granulosum stratum ($p < .0001$) (Figure 1d; Supplementary Table S2), as this stratum was present only in OOM.

SEM analysis of surface differentiation patterns of each type of oral mucosa revealed several differences between samples. As shown in Figure 1e, superficial cells of POM showed evident nuclei, whereas OOM cells were flat and did not show the presence of nuclei. Moreover, higher magnification analysis showed the presence of straight type II microplicae, and curve type III microplicae surface patterns in POM samples, whereas OOM showed type IV and V surface pits (Figure 1e).

3.2 | Immunohistochemical characterization of epithelial components in the human oral mucosa epithelium

When the cytokeratin expression pattern of the oral mucosa epithelium was analyzed by immunohistochemistry (Supplementary Figure S1 and Table 1), we found several differences between both types of samples. First, the analysis using the cytokeratins cocktail AE1/AE3 revealed a strongly positive expression of this marker in all epithelial strata of POM and OOM, although expression was lower at the superficial stratum of OOM. Then, we found that all samples were negative for the marker of simple epithelia CK8. In addition, the immunohistochemical detection of CK5 and CK14 cytokeratins revealed a strongly positive expression at the basal stratum of POM and OOM and a positive expression at the spinosum and superficial strata of POM and at the spinosum stratum of OOM, whereas granulosum and superficial strata of OOM were slightly positive (Supplementary Figure S1 and Table 1). For the keratinization marker CK10, our results revealed a positive expression at the spinosum and superficial strata of POM and a strongly positive expression at the spinosum and granulosum strata of OOM. Analysis of CK13 and CK17 showed that the basal and superficial strata of POM and OOM were negative for both markers, whilst the spinosum stratum of POM and OOM and the granulosum stratum of OOM were positive or strongly positive for these two cytokeratins.

The protein expression analysis of involucrin, a marker of well-differentiated epithelia, showed a strongly positive expression at the spinosum stratum, and a slightly positive signal at the superficial stratum of POM. In OOM, signal was strongly positive at the spinosum and granulosum strata and slightly positive at the superficial stratum. For filaggrin, also linked to epithelial differentiation, we found very low staining signal at the spinosum stratum of POM and a positive signal at the granulosum stratum of OOM (Supplementary Figure S2A and Table 1).

In addition, the immunohistochemical analysis of the intercellular junctions proteins desmoplakin (DSP) and claudin (CLDN) showed pericellular expression in most epithelial cells. In both the POM and OOM, we found a slightly positive signal at the basal stratum and a positive signal at the spinosum stratum, with the superficial stratum being negative for these markers. In the case of OOM, the granulosum stratum was slightly positive (Supplementary Figure S2B and Table 1).

On the other hand, we used Ki67 immunohistochemistry to determine the percentage of epithelial cells showing active cell

proliferation in each type of oral mucosa. As shown in Supplementary Figure S2C,D the percentage of Ki67-positive cells at the epithelial layer was significantly higher in POM as compared to OOM ($p = .0002$) (Supplementary Table S2).

3.3 | Characterization of the human oral mucosa basement membrane

In order to identify the basement membrane in each oral mucosa sample, we used type-IV immunohistochemistry and PAS histochemistry (Figure 2a). Results showed that the basement membrane was positive for both staining methods in POM and OOM, with no differences between these types of samples. Both the POM and OOM showed a well-defined basement membrane between the epithelial and the stromal layers of the oral mucosa.

3.4 | Histochemical and immunohistochemical analysis of ECM components in the human oral mucosa stroma

In the first place, we determined the presence of the main fibrillar components of the oral mucosa stroma using PS, Verhoeff and Gomori's reticulin histochemical methods. For PS, results showed that the stroma of POM and OOM was very rich in collagen fibers, and the collagen network was found anisotropically distributed throughout the stroma (Figure 2c). Quantitative analyzes of PS staining signal revealed that the intensity of the collagen staining signal was significantly higher in OOM than in POM both at the papillar stroma and at the subpapillar stroma ($p < .0001$ for both types of stroma) (Figure 2b and Supplementary Table S2). For Verhoeff and Gomori's reticulin methods, our results show a complete absence of signal in both the POM and OOM, suggesting that elastic and reticulin fibers were not present in the human oral mucosa stroma (data not shown).

In the second place, the analysis of nonfibrillar ECM components using AB showed that OOM contained significantly more abundant proteoglycans at the subpapillar stroma than POM (Figure 2b,c and Supplementary Table S2). ($p < .0001$), although differences between OOM and POM were nonsignificant at the papillar stroma ($p > .05$). Analysis of two specific proteoglycans using immunohistochemistry confirmed that these components tended to be more abundant in OOM than in POM, with significant differences found for versican (at both the papillar and subpapillar stroma, $p < .0001$ in both cases), but not for decorin ($p = 0.0076$ for the papillar stroma and $p > .05$ for the subpapillar stroma) (Figure 2b and Supplementary Table S2).

3.5 | Analysis of blood vessels and cell density at the stromal layer of the human oral mucosa stroma

Analysis of blood vessels showing positive signal for the endothelial marker CD34 revealed that the stroma of both types of oral mucosa contained abundant blood vessels, especially at the papillar area (Figure 3a). However, quantification of these structures revealed that differences between POM and OOM were nonsignificant at both the papillar and the subpapillar areas (Figure 3b and Supplementary Table S2). The same pattern was observed when thick blood vessels showing positive signal for SMA were quantified, with nonsignificant differences between POM and OOM.

Furthermore, we quantified the number of stromal cells found at the stromal layer of POM and OOM after labeling these cells using vimentin immunohistochemistry. As shown in Figure 3c,d and Supplementary Table S2, we found that cells were preferentially allocated at the papillar area of the stroma. When stromal cells were compared between POM and OOM, we found that the number of cells was significantly higher in OOM as compared to POM only at the papillar area ($p = .0002$), and nonsignificant differences were found at the subpapillar area ($p > .05$) (Supplementary Table S2).

4 | DISCUSSION

Despite its important role in the oral cavity (Dawson et al., 2013; Moore, 1972; Sha-Sha et al., 2019), the specific histofunctional features of POM and OOM are not well understood. In this work, we performed a comparative study of both types of masticatory oral mucosae to determine the main characteristics of POM and OOM in the epithelial, basement membrane and stromal compartments using an array of histological, histochemical and immunohistochemical methods that could contribute to determine the main characteristics of each type of tissue. In general, our results show that POM and OOM share many similarities, although specific differences were also found.

As expected, our histological analysis confirmed that both types of mucosae consisted of a well-structured epithelial layer overlying a dense connective stroma, with abundant rete ridges and papillae between both structures (Ciano & Beatty, 2015; de Ferraris & Muñoz, 2023). However, our histomorphological analysis confirmed the presence of nuclei in all strata of POM and the absence of these structures in the superficial strata of OOM, as previously reported (de Ferraris & Muñoz, 2023). Furthermore, we found that the epithelium was significantly thicker in POM, and that these differences were mainly due to the presence of a more developed spinosum stratum, with no differences in basal and superficial strata. Although previous reports demonstrated that the stratum spinosum is the thickest stratum of the oral mucosa epithelium (Adams, 1976; Sha-Sha et al., 2019), these differences between POM and OOM had not been reported to the date. Similar results were previously reported by our group for the human skin, where we found significant differences when palmar and plantar skin was compared to dorsal skin of hands and feet (Vela-Romera et al., 2019).

In the case of the oral mucosa, the higher number of cells at the stratum spinosum of POM could imply that this type of masticatory oral mucosa could be subjected to stronger requirements as protective tight barrier, as cells in this stratum are characterized by the presence of abundant intercellular junctions (Groeger & Meyle, 2019). In this regard, it has been demonstrated that the

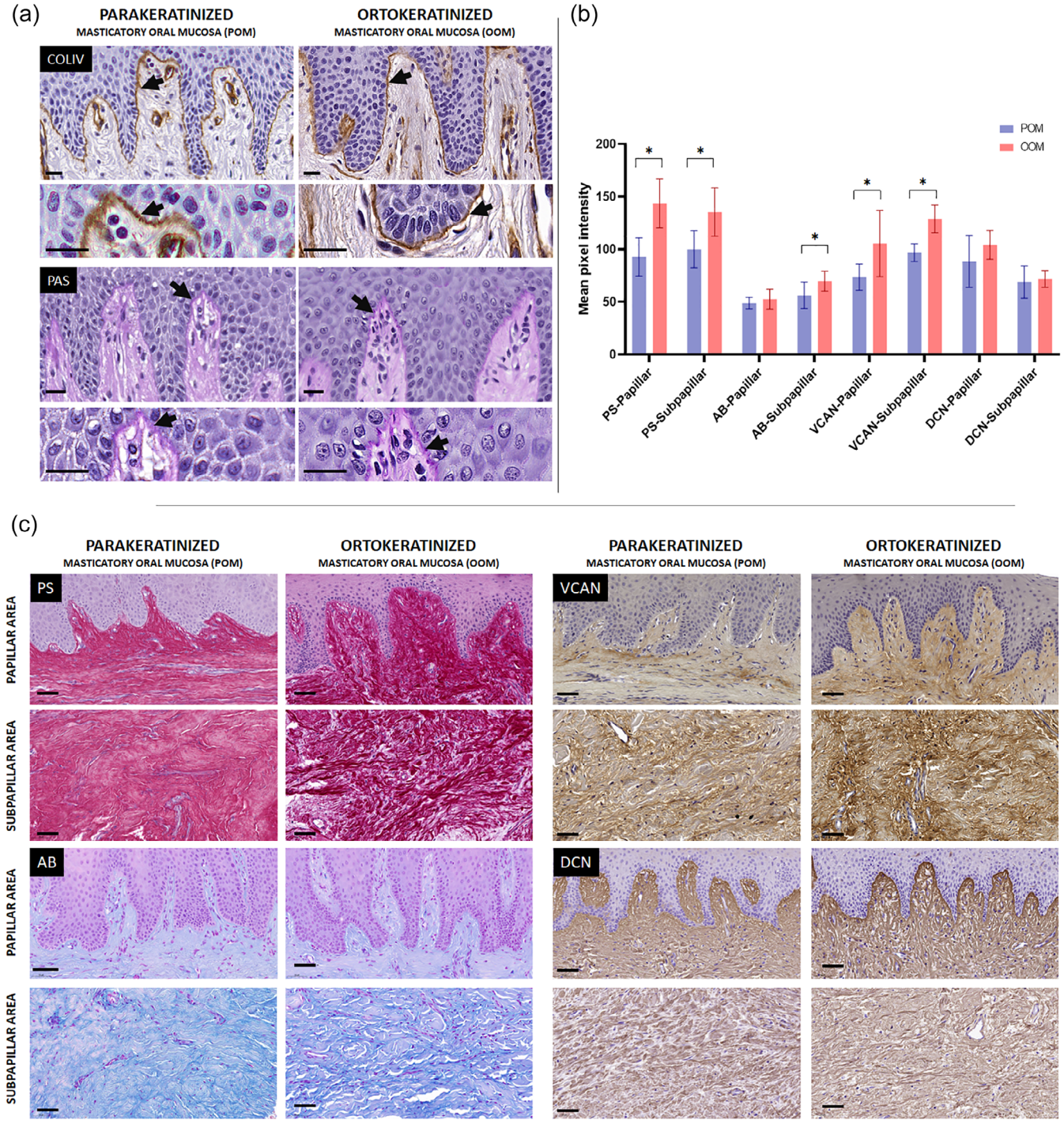

FIGURE 2 Analysis of basement membrane and extracellular matrix (ECM) components at the stromal layer of parakeratinized (POM) and orthokeratinized (OOM) masticatory human oral mucosa. (a) basement membrane assessment by type-IV collagen (COLIV) immunohistochemical analysis and by carbohydrates and glycoproteins histochemical analysis with periodic acid–Schiff (PAS) staining. Arrows show illustrative areas of positive staining signal for each analysis method. (b) and (c) showed the quantitative and histological analysis of the ECM components at the stroma layer. In (b) the quantitative analysis of the positive staining reaction of the ECM components were analyzed in each area of POM and OOM. Statistically significant differences between POM and OOM are highlighted with an asterisk (*). In (c) collagen fibers and proteoglycans were identified by picrosirius (PS) and alcian blue (AB) histochemical methods, respectively, at the papillar and subpapillar areas of the stroma, and versican (VCAN) and decorin (DCN) proteoglycans identification using immunohistochemistry. Scale bar = 20 μm in (a) and 50 μm in (c).

normal oral microbiome associated to the human oral mucosa has regional differences (Uzunoğlu et al., 2021), and we might hypothesize that regions subjected to mild masticatory forces covered by POM could be colonized by more abundant microbial populations, whereas regions covered by OOM could be associated to more scarce populations due to continuous squamation associated to

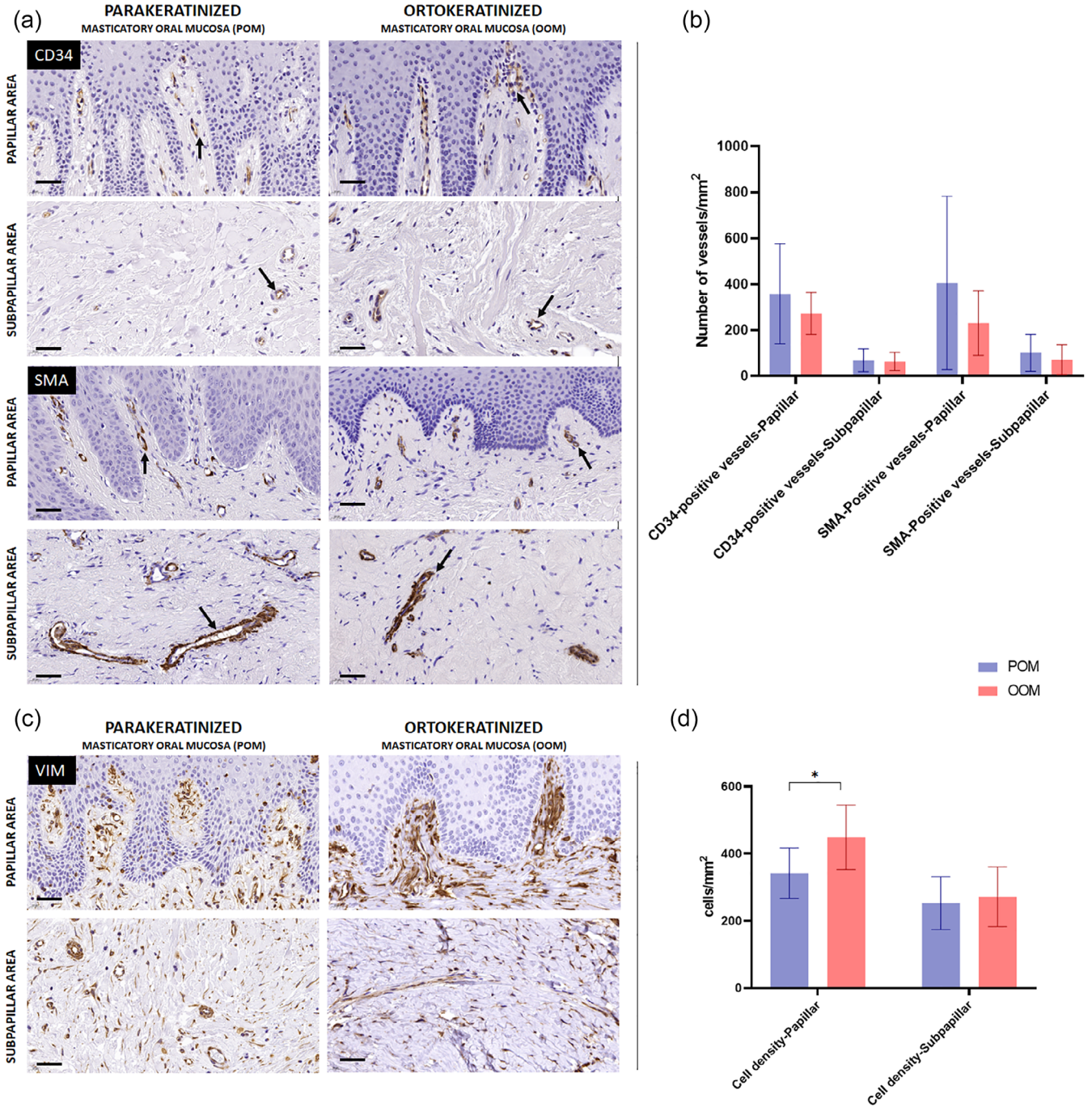

FIGURE 3 Immunohistochemical analysis of blood vessels and cell density in the stromal layer of parakeratinized (POM) and orthokeratinized (OOM) masticatory human oral mucosa. (a) Analysis of blood vessels showing positive immunohistochemical signal for CD34 and SMA markers. Arrows highlight illustrative positive vessels at the papillar and subpapillar areas. (b) Quantitative study of the number of blood vessels per mm^2 of stroma in papillar and subpapillar areas of POM and OOM. (c) Identification of stromal cells showing positive immunohistochemical staining signal for vimentin. (d) Quantitative study of the number of vimentin-positive cells per mm^2 of stroma. Statistically significant differences between POM and OOM are highlighted with an asterisk (*). Scale bar = 40 μm.

mastication. Future works should confirm or not these results. Interestingly, our analysis of the presence of intercellular junctions did not reveal any differences between POM and OOM, which could imply that both types of tissue contain cells joined by strong cell–cell junctions, and the difference between both types of oral mucosae is the thickness of the stratum spinosum, but not the structure or composition of this stratum. These findings, along with our results showing that the size of the cells was similar in all epithelial strata, is in agreement with previous works suggesting that POM and OOM are structurally similar, and their cells contain the same structures and cell organelles, although some differences could exist regarding their content of tonofilaments (Adams, 1976).

Moreover, our analysis of cells showing positive Ki67 expression did found significant differences, with POM containing a higher percentage of proliferating cells than OOM. These results would correlate with the higher number of cells found in POM, and the higher turnover requirements of this type of tissue, as a relationship between function and proliferative activity has been previously demonstrated in several types of epithelia (Hill et al., 1981).

Interestingly, we also found that POM was devoid of a well-defined stratum granulosum, whereas this stratum was clearly identifiable in OOM, as previously reported (Sawa et al., 2021). It is well known that the stratum granulosum is a previous step toward terminal keratinization and squamation, and the presence of this stratum is strictly associated to orthokeratinized strata. Although the absence of the stratum granulosum was not previously reported in POM, it has been demonstrated that OOM subjected to cryotherapy experienced a process of histological reversion to POM, and this modification was associated to a modification and increase of the stratum granulosum, which became comparable to OOM (Tal et al., 1982). In addition, it has been reported that orthokeratinized epithelia contain highly electrodense keratohyalin granules, which are typical of the stratum granulosum, whereas other types of epithelia contain other types of granules (Moore, 1972). These findings are in agreement with the presence of type IV and V surface differentiation patterns in OOM, since these patterns are typically associated to the surface terminally differentiated epithelia, especially in the case of orthodifferentiated epithelia, whilst other types of oral mucosa epithelia usually show other types of surface patterns (Adams, 1976; Alaminos et al., 2007; de Ferraris & Muñoz, 2023; Moreu et al., 1993; Worawongvasu, 2007).

After characterizing the oral mucosa epithelium at the histomorphological level, we carried out an immunohistochemical analysis of the main cell markers found at this site, and we found that the epithelium of POM and OOM expressed high amounts of cytokeratins, as expected. However, several differences were found when specific cytokeratins were analyzed. Cytokeratins are intermediate filament proteins playing important roles in a number of epithelial cell functions, including cell differentiation, cell–cell adhesion, resistance to stress forces and formation of a functional protective barrier (Jacob et al., 2018; Kuburich et al., 2022; Szeverenyi et al., 2008), and their alterations have been associated to different epithelial diseases. In our study, we found that cytokeratins were expressed by all strata of the oral mucosa epithelium, especially by the spinosum and granulosum strata. As expected, we found that both POM and OOM were devoid of CK8 expression at all epithelial strata. CK8 is a marker of simple epithelia, and is normally expressed only by the human urothelium (Gaston & Grossman, 2010), epididymis (Pinel et al., 2019) and other types of simple epithelia. However, the other types of cytokeratins analyzed here showed to be positive in POM and OOM, although some expression patterns differed between both types of samples.

When the basal stratum was analyzed, we found a positive immunostaining signal for cytokeratins AE5/AE3, CK5 and CK14, and a negative signal for other types of cytokeratins, with no differences between POM and OOM. Apart from AE1/AE3, which is an unspecific cocktail of cytokeratins, CK5 and CK14 are typically expressed by proliferating epithelia, and have been used as a marker of keratinocytes (Bucchieri et al., 2012; Thomsen et al., 2022). Their expression at the basal stratum, along with the absence of other cytokeratins that are associated to keratinocyte differentiation, confirms the stemness character of cells allocated at this stratum, and suggests that no differences between POM and OOM may exist at this level.

Regarding the stratum spinosum, our cytokeratin expression analysis revealed that this layer was functionally very similar in POM and OOM. In fact, spinosum keratinocytes expressed all the evaluated cytokeratins, except CK8, and the expression pattern was very similar in both cases. The only exception was CK10, which was more intensively expressed by OOM as compared to POM. These results are not surprising, since CK10 is known to be a mediator of terminal keratinocyte differentiation that is present in suprabasal layers of the human oral mucosa, and its presence is fundamentally associated to orthokeratinized epithelia subjected to strong biomechanical requirements (Bauer et al., 2012). In turn, expression of CK13 and CK17 in this layer is an expected finding, as these markers are found in normal human oral mucosa. In fact, CK13 is typically expressed by this tissue, and plays a role in maintaining the homeostasis of the different strata of the epithelium. Its alterations are associated with the presence of different types of disfunctions of the human oral mucosa epithelium (Qiao et al., 2022). Similarly, CK17 is normally found in the oral mucosa epithelium, and its overexpression and dysregulation has been described as a diagnostic marker of several diseases, including the oral squamous cell carcinoma (Kitamura et al., 2012).

Additionally, our analysis of the stratum granulosum found in OOM showed very similar cytokeratin expression patterns than POM, and the only difference was a lower expression of CK5 and CK14, markers of undifferentiation, stemness and active proliferation. These results would confirm that the stratum granulosum is functionally similar to the stratum spinosum, with the difference that granulosum keratinocytes would have lower proliferation capability and higher differentiation degree than spinosum keratinocytes.

Finally, the superficial stratum of the oral mucosa epithelium showed several dissimilarities between both types of oral mucosae. While the superficial stratum of POM expressed AE5/AE3, CK5, CK14 and CK10, OOM showed lower expression levels of all these cytokeratins. In this sense, it has been demonstrated that the stratum corneum of terminally differentiated epithelia mainly consists of several layers of corneocyte cells containing abundant lipids (Dawson et al., 2013), in which the cytoskeletal network experienced a structural collapse that is associated with the aggregation of keratins and the disruption of intracellular proteins into basic amino-acids (Rawlings et al., 1994). For this reason, the stratum corneum of the human oral mucosa typically shows lower expression of some cytokeratins than other strata of the same epithelium, as previously found in the human skin (Vela-Romera et al., 2019).

Other typical markers of well differentiated epithelia are filaggrin and involucrin, which are associated to terminal differentiation and play a crucial role in the formation of a structural protective barrier by differentiated epithelia (Furue, 2020). Its presence in both the POM

and OOM would contribute to protect the inner tissues from the potentially harmful environment found at the oral cavity (Dawson et al., 2013; Sha-Sha et al., 2019). In our study, we found no differences for involucrin expression. However, filaggrin was positively expressed by the granulosum stratum of OOM, and expressed at very low levels by the upper layers of the stratum spinosum of POM. Cells in the stratum granulosum are known to contain abundant keratohyalin granules mostly composed by filaggrin and profilaggrin (Furue, 2020), and the intense expression of filaggrin has been previously reported at the stratum granulosum of human epithelia (Ishitsuka & Roop, 2020; Makino et al., 2016).

Moreover, our study of the basement membrane of POM and OOM revealed no differences between both types of oral mucosae. It has been demonstrated that the presence of a well-differentiated basement membrane is crucial for a proper epithelium physiology and attachment to the underlying connective tissue (Breitkreutz et al., 2009). The fact that both types of masticatory oral mucosae had a well-structured basement membrane could be associated with the need of the epithelium subjected to masticatory forces to firmly attach to the stromal layer and prevention of microorganism invasion (Salonen et al., 1984). In fact, the epithelial-mesenchymal interaction mediated by the basement membrane is fundamental for the development and maintenance of a functional oral mucosa (Liu et al., 2011; Rich & Reade, 2001; Sharpe & Ferguson, 1988).

Besides the epithelial layer and basement membrane, the stroma is a crucial component of the human oral mucosa. This layer provides structural and physiological support to the epithelium and is partially responsible for the human oral mucosa barrier function (Kullage et al., 2017; Omori et al., 2021). In this study, we found some differences in the stroma layer of POM and OOM. Regarding the fibrillar components of the oral mucosa stroma, our results found significant differences for the presence of collagen, which was more abundant in OOM than in POM. These fibers play a crucial role in controlling the biomechanical properties of human tissues, and its presence is normally associated to increased resistance and resilience to incoming forces (Chang & Buehler, 2014; Silver et al., 2021). Although specific biomechanical analysis should confirm this hypothesis, our finding that OOM was enriched in fibrillar collagen fibers in both the papilllar and subpapilllar regions, may suggest that OOM stroma could have higher resistance to physical aggressions that POM, which is in concordance with the specific functions of each type of tissue.

On the other hand, proteoglycans are important nonfibrillar components of the human ECM, as they can interact with other components, such as collagen fibers, to form a meshwork that provides tensile strength to enhance the physical resistance of the tissue (de Mattos Pimenta Vidal et al., 2017; Silver et al., 2021). In the present study, we found that OOM contained more proteoglycans identified by AB at the subpapillar area than POM, although no differences were detected at the papillar area. Again, these results could be explained by the higher biomechanical requirements of OOM as compared to POM and its higher capability to support physical forces (Silver et al., 2021). Moreover, we found that versican was more abundant in the stroma of OOM as compared to POM. Versican are negatively charged proteoglycans able to attract positively charged ions, such as sodium ions, thus creating an osmotic gradient that attracts water molecules into the ECM, providing the stroma with a gel-like consistency able to resist compression forces and to absorb shock forces (Orgel et al., 2009).

Another intriguing finding of our study was the higher presence of stromal cells at the papillar stroma of OOM as compared to POM. In this regard, it is important to note that vimentin is a marker able to label stromal fibroblasts, but also other types of cells present in the stroma, including macrophages, plasma cells, mast cells and lymphocytes, which play a role in protecting the oral mucosa from external pathogens that may enter the mucosa (Maquart & Monboisse, 2014; Waasdorp et al., 2021). Although the higher number of stromal cells found in OOM could be related to the development of a more mature and complex ECM found in this type of masticatory oral mucosa, it is also possible that the thinner epithelium found in OOM could be associated with an increased need of immune-related defense cells to protect from microorganisms able to reach the stromal layer of the oral mucosa.

Furthermore, blood vessels are very important components of the oral mucosa stroma, as they provide oxygen and nutrients and are directly related with tissue homeostasis, repair, and regeneration (Naumova et al., 2013). In this study, we found that the oral mucosa stroma was enriched in thin blood vessels detected by CD34 immunohistochemistry, and in thicker vessels containing a muscular wall detected by SMA immunohistochemistry. Although the papillar region tended to show higher concentration of both types of structures, no differences were found between POM and OOM, suggesting that both types of tissues are very well irrigated, and the availability of oxygen and nutrients could be similar in both cases.

The selection of appropriate microscopy methods is crucial in elucidating the histological, cellular, and molecular characteristics of tissues. In this study, we employed a multidisciplinary approach combining light microscopy analysis with histological, histochemical and immunohistochemical techniques and SEM to identify specific markers in POM and OOM. This approach enabled us to visualize and assess the location, intensity, and distribution of these markers, providing valuable insights about the cellular composition and differentiation processes in the epithelium and stroma. On the one hand, the use of SEM allowed us to identify specific three-dimensional surface patterns that cannot be detected using other microscopical methods. On the other hand, light microscopy was used to characterize the structure of the main components of the human masticatory oral mucosa, whereas specific histochemical and immunohistochemical methods provided valuable information on the composition and histofunctional features of these tissues. Altogether, the combinatorial use of these techniques enabled the in-deep characterization of the human masticatory oral mucosa. The integration of different microscopy techniques, alongside the inclusion of relevant images, enhanced the visual representation and clarity of our findings and provided a comprehensive approach to studying the oral mucosa, contributing to understand its normal histoarchitecture and function, and providing a solid foundation for future investigations in pathological conditions.

In summary, the present work demonstrated that POM and OOM share many similarities from a histological standpoint. However, important differences exist both at the epithelial and stromal levels, and these differences could be related to the different biomechanical requirements of both types of tissues. These results could contribute to a better understanding of the pathological conditions affecting the human masticatory oral mucosa, such as different types of neoplasia (Karantza, 2011). In addition, these findings could be useful for the generation of different types of oral mucosae by tissue engineering, as previously reported (Alaminos et al., 2007). Future works should be carried out to confirm the present results at the genetic and biochemical levels.

AUTHOR CONTRIBUTIONS

Miguel Ibáñez-Cortés: Data curation; investigation; methodology. **Miguel Ángel Martin-Piedra:** Conceptualization; methodology; investigation. **Cristina Blanco-Elices:** Data curation; methodology; investigation. **Óscar Darío García-García:** Data curation; formal analysis; visualization. **Antonio España-López:** Visualization; investigation; conceptualization. **Ricardo Fernández-Valadés:** Conceptualization; visualization; methodology. **María del Carmen Sánchez-Quevedo:** Conceptualization; writing – review and editing; supervision. **Miguel Alaminos:** Writing – review and editing; supervision. **Jesús Chato-Astrain:** Writing – original draft; methodology; validation. **Ingrid Garzón:** Project administration; funding acquisition; supervision.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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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