Labial Salivary Gland Biopsy in Sjögren's Syndrome

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Introduction

Labial salivary gland (LSG) biopsy plays a fundamental role in the diagnosis and in the 2002 AECG and 2016 ACR/EULAR classification of primary Sjögren's syndrome (pSS) [1, 2]. Moreover, a potential role as the predictor of disease severity and biomarker in clinical trials has been recently hypothesized [3]. To achieve these clinical end points, several steps should be correctly followed: from the LSG biopsy procedure, through an adequate processing of the tissue, to the final histopathological interpretation. In this chapter, we will focus on three main aspects: i) the LSG biopsy procedure; ii) the histopathological evaluation; iii) the performance of histopathology as a diagnostic and prognostic tool. The target users include histopathologists, rheumatologists, and specialists in oral and ocular diseases.

The LSG Biopsy Procedure

LSG biopsy was first popularized by Chisholm and Mason [4] and is based on the assumption, verified by several studies, that LSG biopsy and parotid biopsy can be generally comparable for the diagnosis of pSS [5, 6]. Although operative variations have been described over time, general principles of the LSG biopsy procedures are similar [7].

The procedure starts by everting the inferior lip to allow maximum exposure of the mucosal lining. This maneuver can be performed using a chalazion clamp [8, 9], although a bimanual approach is also effective and less aggressive (Fig. 13.1a). An imaginary incision line of about 1 cm is drawn horizontally (Fig. 13.1b) which is then infiltrated with a lidocaine-adrenaline solution (Fig. 13.1c). The mucosal lining is incised with gentle pressure using an n.11 scalpel blade (Fig. 13.2a). Appropriate hemostasis is performed by softly cleaning across the incision margins. Electrocautery should be avoided in this phase in order not to cause necrosis of the underlying salivary glands, thereby reducing the number of harvested glands. Gentle undermining of the submucosal layer is performed on both sides of the incision using blunt-tipped scissors to mobilize the mucosa over the submucosal layer containing the minor salivary glands. The result is a clear exposure of the glands, which protrude from the incision site (Fig. 13.2b). In addition, caution should be paid not to dissect the lip branches of the mental nerve.

Using delicate forceps, e.g., the Adson's forceps, incision margins are everted for the exposure of glands (Fig. 13.3a). Glands tend to be visible as pale, pinkish globular structures and are generally well represented, although in some circumstances one single incision does not allow harvesting all the required number of glands, and another site has to be located. Glands are gently grasped using Adson's forceps and sectioned at the base (Fig. 13.3b, c). Dissection above the muscular plane is performed with blunt tipped scissors along a vertical axis, which is parallel to the direction of sensory nerves. It is very important to obtain enough gland tissue which, according to a recent consensus, is suggested to be four glandular lobules [10], although in our experience six-to-eight lobules represent the preferred choice.

Gland lobules are collected in a container with formalin and sent for final histopathologic examination (as subsequently described). Any bleeding source is identified and selectively controlled using a needle tip electrocautery. Wound margins are approximated for optimal closure; eventually, two single hooks can be placed at the extreme points and gently tractioned to assess the optimal alignment of

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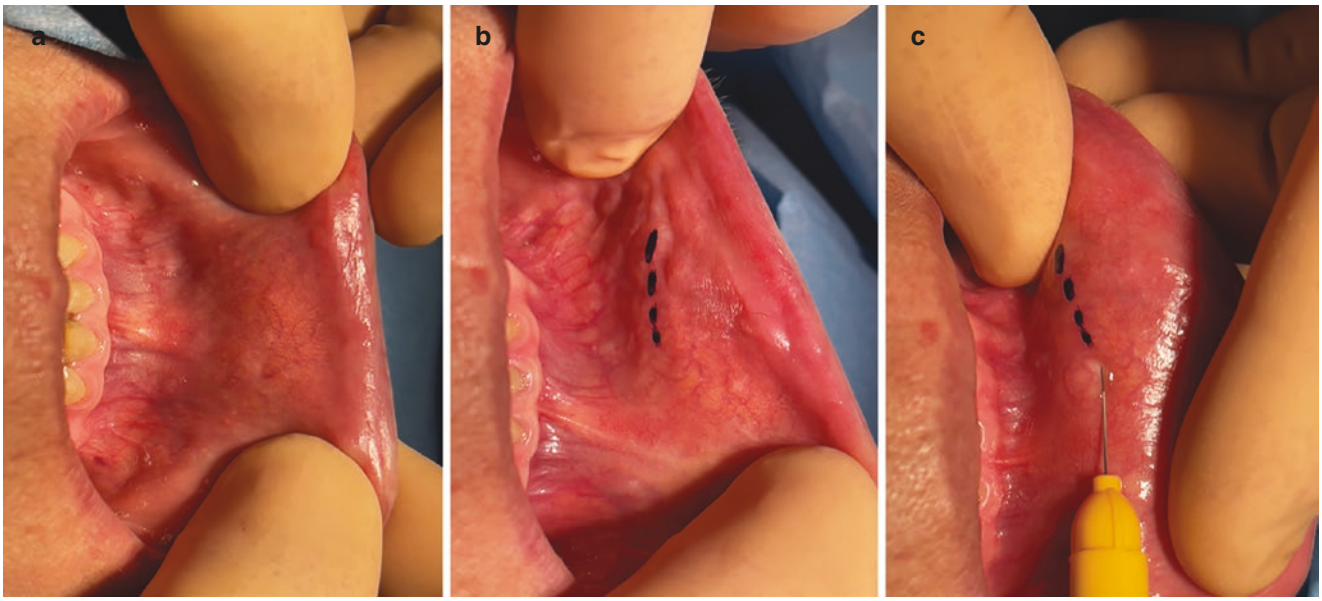

Fig. 13.1 (**a**) The inferior lip is everted bimanually for the optimal exposure of the mucosa; (**b**) the incision line is drawn; (**c**) infiltration with a local anesthetic and a vasoconstrictor is performed along the incision line

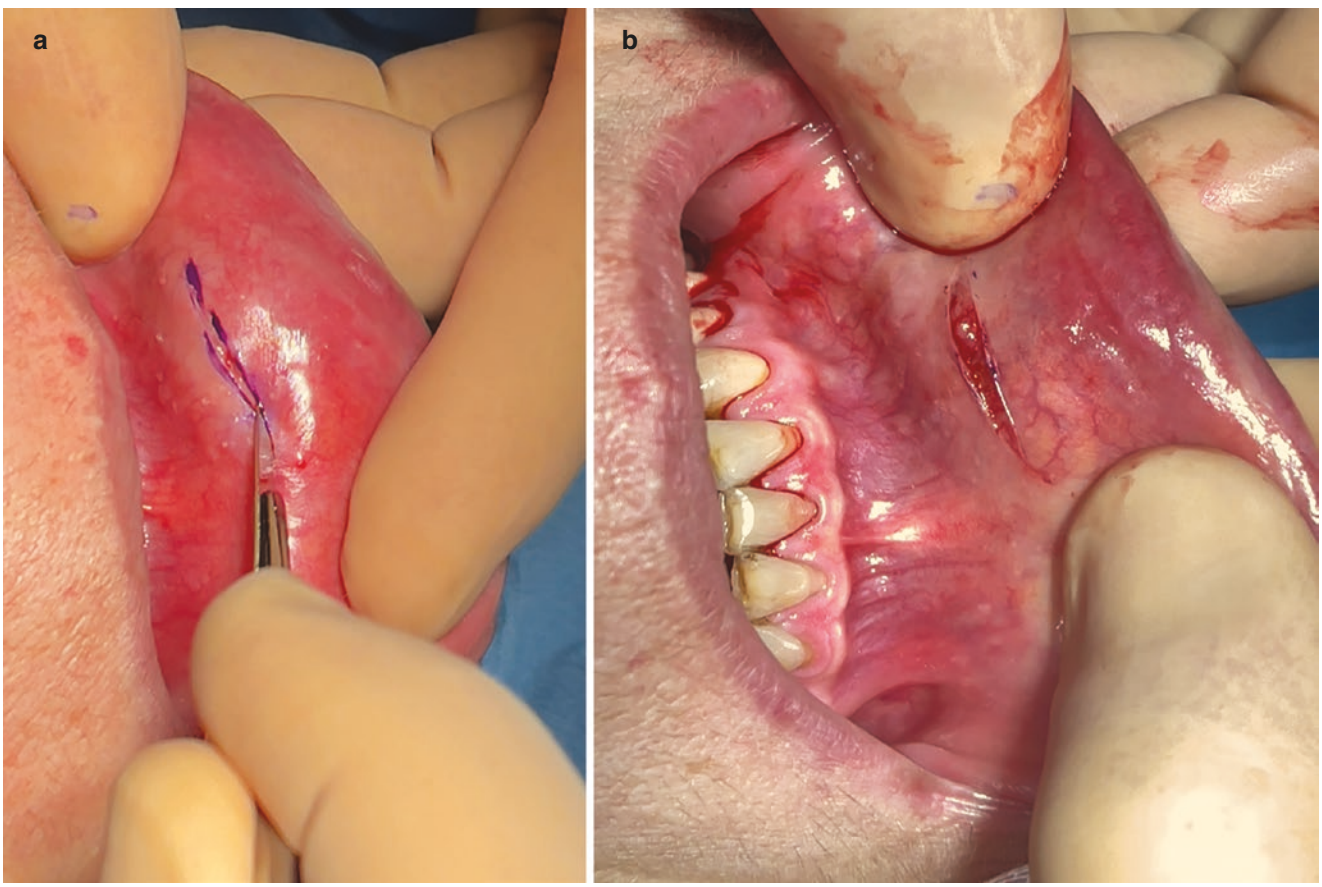

Fig. 13.2 (**a**) A scalpel is used to incise the mucosa along the defined line; (**b**) once the mucosal layer is incised, minor salivary glands are immediately visible

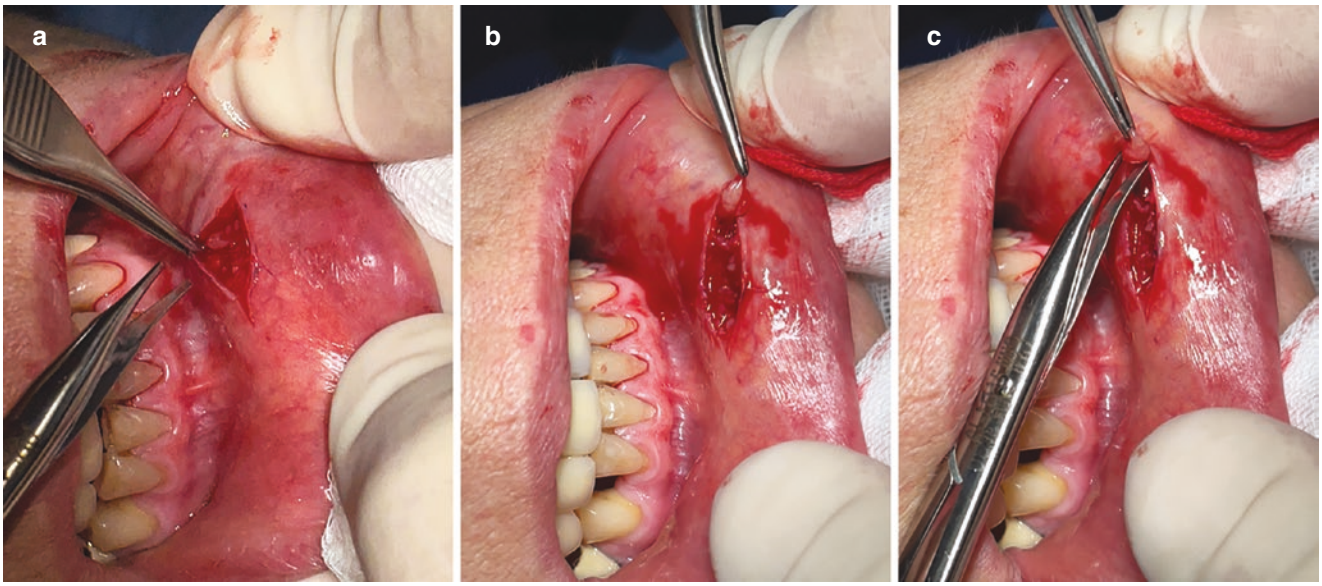

Fig. 13.3 (**a**) An Adson's forceps is used to grasp the mucosa; (**b**) salivary glands are pulled up and (**c**) sectioned at the base

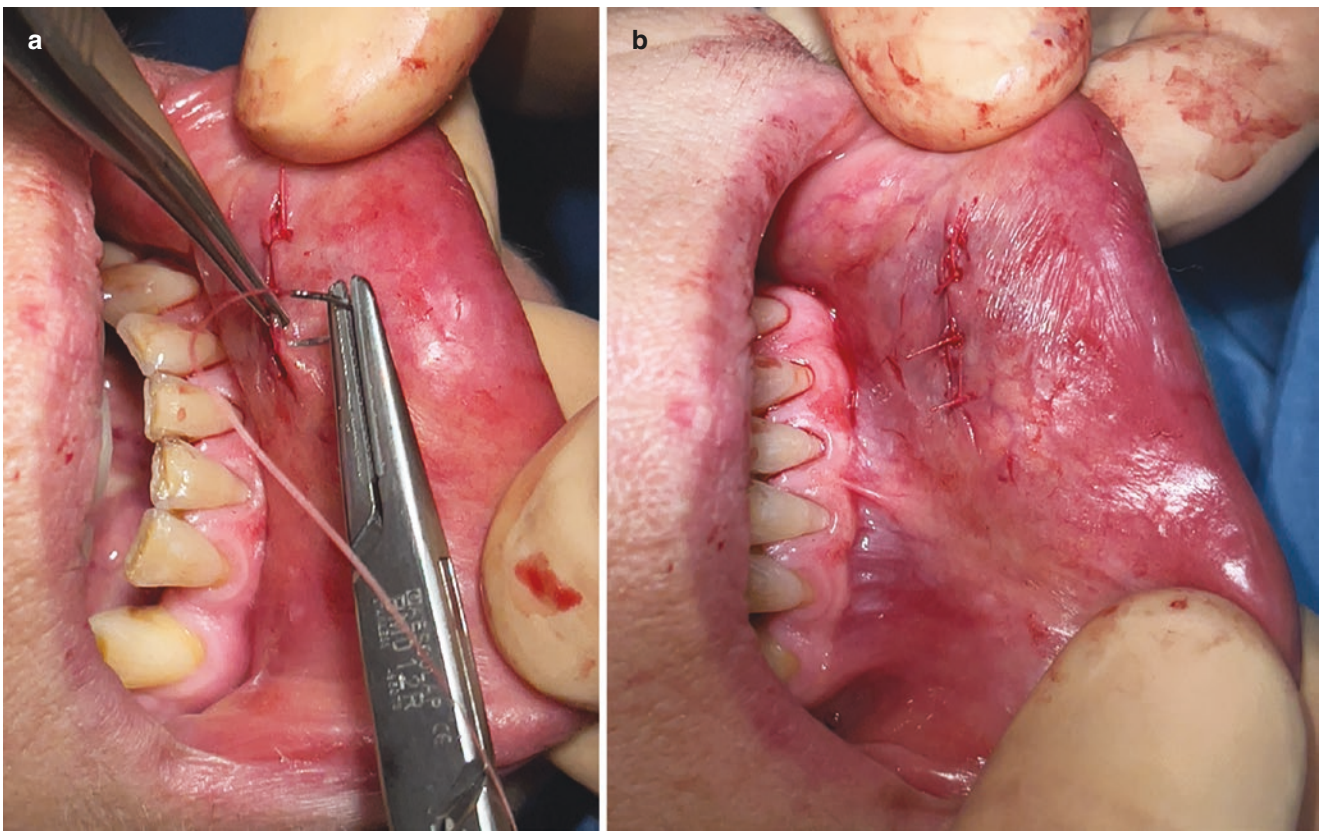

Fig. 13.4 (**a**) a simple suture is placed to close the wound; (**b**) overview of the final suture

mucosal surfaces. Wound closure is performed using absorbable 4–0 single sutures placed on the mucosal layer (Fig. 13.4a, b).

LSG biopsy is not devoid of complications, which have been reported in the literature [11]. The most common are sensory alteration of the lower lip which patients often report as "numbness" sensation, local pain, swelling, bruising, hematoma, and wound infection [10, 12]. The above-described sensory alteration can be permanent in up to 10% of cases [7].

The LSG Histopathological Evaluation

The Pathology Workflow

The pathology workflow is usually divided into three phases: pre-analytic, analytic, and post-analytic one, each consisting of a series of intermediate steps. In particular, the pre-analytical phase, which includes specimen handling issues occurring prior to the arrival time in the laboratory, is a crucial step in the pathology workflow. To preserve tissue morphology and tissue antigenicity for immunohistochemical and molecular exams, the LSG biopsy material must be readily placed in an adequate amount of fixative (usually 10% neutral buffered formalin); moreover, the fixation time has to be controlled and standardized in order to avoid under- or over-fixation issues; both of these conditions can affect the final diagnosis if ancillary tests are applied. In particular settings (if other exams are planned to be performed, especially for research purposes, or in centers where a tissue biobank is established), the biopsy material can be sent fresh to the pathology department where part of the fragments will be snap-frozen and part processed for routine histology. Fresh tissue can also be sent for flow cytometry if a hematological disease, although rare in this site, is suspected. After fixation, the tissue fragments are routinely processed, embedded in paraffin (care should be given to preparation of paraffin blocks, with smaller glands set higher) [10], cut in 3.5–4 μm thick sections, placed on a glass slide and stained with Hematoxylin & Eosin (H&E).

The Histopathological Evaluation of LSG

The primary function of the salivary glands is to moisten the mucous membranes of the upper aerodigestive tract. In humans, this function is fulfilled by the continuous secretion of numerous minor salivary glands. These glands, ranging in size from 1 to 5 mm, are located in the submucosa throughout the oral cavity, pharynx, and upper airways with the greatest density in the lips, tongue, buccal mucosa, and palate. Salivary glands are defined as exocrine glands that secrete saliva through ducts from a secretory structure called the salivary acinus; the acinus itself can be divided into three main types: serous, mucinous, and mixed. Serous acini in salivary glands are roughly spherical and are composed of pyramidal cells, with basally located nuclei surrounded by dense basophilic granular cytoplasm and secretory granules. On the other hand, mucinous acinar cells are commonly simple columnar cells with flattened, basally situated nuclei and water-soluble granules that make the intracellular cytoplasm appear clear. Mixed, or seromucous, acini contain components of both types, but one type of secretory unit may dominate. The majority of LSGs are either mucinous or seromucinous. Between the epithelial cells and basal lamina of the acinus lies the flat myoepithelial cells network that, with contraction, can force secretion of the acinus. The other important component of the salivary gland parenchyma is the salivary gland duct system. The acini first secrete through small canaliculi into the intercalated ducts, which in turn empty into striated ducts within the glandular lobule and then into the interlobular excretory ducts [13, 14] (Fig. 13.5a, b).

The Histology of LSG in pSS

The histopathological hallmark of pSS is the focal lymphocytic sialadenitis (FLS), characterized by the presence of lymphoid foci in a periductal or perivascular glandular localization [10, 15]. A lymphoid focus is defined as a dense aggregate of at least 50 mononuclear cells, usually placed around ducts (striated or intercalated) or vessels, while the surrounding tissue is mainly composed of unaffected parenchyma (Fig. 13.5c). Foci are composed of T and B lymphocytes, the former prevalent, arranged in a non-segregated manner, with plasma cells aligned at the periphery [10]. The Focus Score (FS) is calculated by dividing the number of foci by the total glandular surface area in mm^2 multiplied by 4 to yield the number of foci per 4 mm^2. A FS $\geq$1 per 4 mm^2 is considered as a positive biopsy and used for the classification of pSS, according to the 2002 AECG and 2016 ACR/EULAR criteria[1, 2].

The FS ranges from 0 to 12: above a FS of 10, foci are typically confluent and a maximum score of 12 is applied [16].

It is currently recommended that the presence of FLS should be determined prior to FS calculation.

Given the scattered nature of foci, it is important that there is sufficient material available to allow a robust and reliable analysis: a minimum glandular surface area to be examined of 8 mm^2 is required [17], since the examination of an insufficient glandular area may lead to an underestimation or overestimation of the FS. The glandular tissue, as mentioned above, should be well preserved and devoid of cutting artifacts; the whole of the glandular surface area in the denominator should be included. If the glandular tissue is below the limit, two additional cutting levels at 200 μm intervals should be obtained [10]. No data are available regarding how measurements are carried out except by means of an eye piece grid or, more precisely, by a measurement-validated microscope-associated software [10].

Difficulties in interpretation may arise when pSS features are associated with non-specific chronic sialadenitis (NSCS), such as acinar atrophy, interstitial fibrosis, and duct dilata-

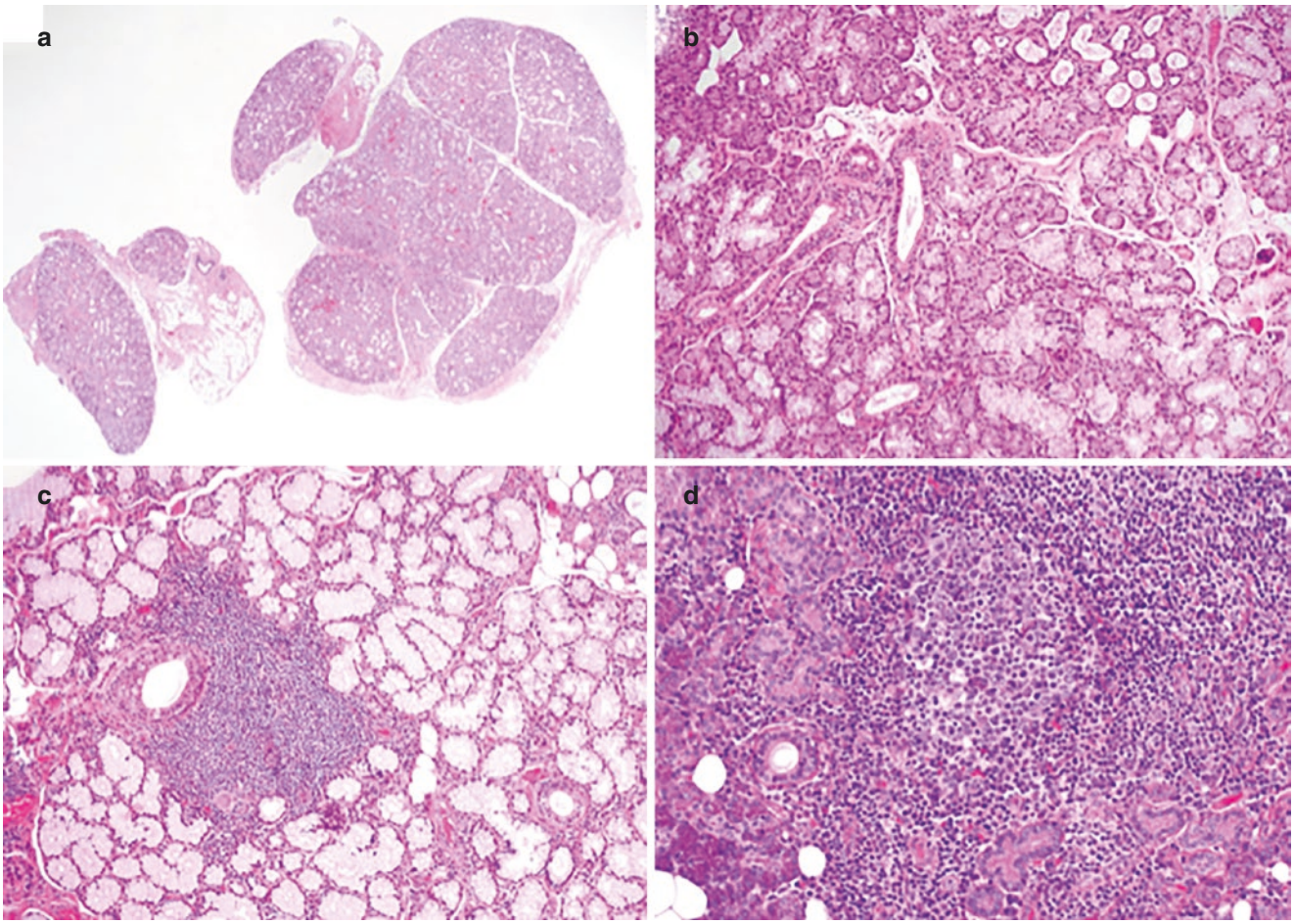

Fig. 13.5 Hematoxylin and eosin stained sections of labial salivary gland (LSG). (**a**) Low magnification of normal LSG lobules (H&E stain; original magnification ×20); (**b**) higher magnification showing normal LSG (H&E stain; original magnification ×100); (**c**) a lymphoid focus in periductal location from a patient with focal lymphocytic sialadenitis (H&E stain; original magnification ×100); (**d**) a dense lymphoid infiltrate with MALT acquisition and formation of a germinal center (H&E stain; original magnification ×200)

tion, i.e., relatively common abnormalities that increase with age and therefore may coexist with pSS. Moreover, NSCS itself may be accompanied by infiltration and even foci of lymphocytes, thus raising issues for interpretation [16].

When present, the extent of the atrophic features should be reported and graded (e.g., absent, mild, moderate, severe) to aid the referring clinician in their interpretation.

With the progression of pSS, secondary lymphoid follicles and lympho-epithelial lesions (LEL) may be observed within foci zones, sometimes with activated germinal centres (GC). The structures are defined as "segregated," characterized by a GC with follicular dendritic cells, a mantle zone, a possible marginal zone, circumscribed by a T cell area containing high endothelial venules; this histopathological picture is referred to as MALT acquisition [18] (Fig. 13.5d).

Unfortunately, in the literature no mention is made of the development of secondary lymphoid follicles within or adjacent to foci, in the calculation of the FS; however, the presence or absence of GC should be reported, as they represent possible predictors of lymphoma development, although their role needs to be clarified with further research [16, 19–22].

Another histological feature that can develop in pSS patient and must be reported is the presence of the so-called lympho-epithelial lesions (LELs) or epi-myoepithelial islands, characterized by a proliferation of the ductal epithelium and myoepithelium, ultimately obliterating duct lumina, associated constantly with intra-epithelial lymphoid exocytosis and adjacent foci or rim of lymphocytes. A few studies suggest that these islands do not contain a myoepithelial component but are composed of metaplastic intercalated ducts with an altered immunophenotype [23].

In pSS biopsies, also a diffuse lymphocytic infiltrate may be present. Chisholm and Mason [4] defined the infiltrate as a mixed one, composed of lymphocytes and plasma cells in non-periductal sites, frequently dispersed at the periphery of lobules.

In addition to the H&E stained section, a small panel of immunohistochemical markers may be performed in routine practice, in order to better evaluate the inflammatory infiltrate. Staining for CD3, CD20, and CD21 markers should be included; and the presence of germinal center-like structures should be reported as the proportion of foci with both T/B-cell segregation and follicular dendritic cell networks [10].

The Role of LSG as a Diagnostic and Prognostic Tool in pSS

In the past [1] as well as in the most recent 2016 ACR/EULAR classification criteria [2], a positive LSG biopsy (i.e., FS ≥ 1 per 4 mm^2) and the presence of anti-SSA antibodies are the most important points to reach a positive score for the classification of pSS. According to the last criteria [2], a positive LSG biopsy accounts for 3 points, with a total score of ≥4 to meet the criteria for pSS.

Overall, if the utility of LSG biopsy examination is surely undeniable; there are many technical, conceptual, and interpretative issues still to be deepened.

According to the systematic literature review of Guellec et al. based on 9 selected studies, LSG biopsy sensitivity ranges from 63.5% to 93.7%, while its specificity comprises between 61.2% and 100%, when compared to expert opinion or classification criteria fulfillment [24]. The positive predictive value and negative predictive value range from 74.2% to 100%, and 39.1% to 96.1%, respectively [24]. The application of the FS is not devoid of miscalculation and failure of correct interpretation. Important flaws of the FS are represented by the evaluation of the number of foci without considering their size and distribution; also the presence of larger areas of acinar atrophy, interstitial fibrosis, and histological patterns as NSCS or sclerosing chronic sialadenitis can lead to interpretative discrepancies [25].

In 2002, Vivino et al. described the results of a blinded expert re-examination of 60 LSG biopsies from 58 patients, requested by various institutions for a second opinion on pSS diagnosis. This review led to a change of initial diagnosis in 53% of patients, generating 22 false-positive or false-negative test results in the diagnosis of pSS, with an error rate of 37%. As 58 of 60 specimens submitted for first diagnoses were unaccompanied by the FS, an failure to employ the FS system was identified as the main cause of misleading [26].

Costa et al. in 2014 assessed intra-observer and inter-observer reliability of LSG biopsy, focusing on FLS, dichotomized FS, and other nine histopathological features (e.g., acinar depletion, fibrosis, adiposis, GC-like structures)[27]. The authors highlighted a substantial intra-observer agreement and a moderate inter-observer reliability for FLS, while dichotomized FS (i.e., scoring values <1 as 0 and values ≥1 as 1) presented an almost perfect intra-observer and a substantial inter-observer agreement. The inter-rater reliability of the other histopathologic features ranged from poor (e.g., duct dilatation, kappa = −0.12) to almost perfect (e.g., adiposis, kappa = 1) agreement.

Thus, to avoid the risk of misclassification and improve the diagnostic accuracy of LSG biopsy, various measures have been proposed, including a strict application of the FS [25], multilevel examination of tissue specimens [28], and more importantly the determination of standardized guidelines developed by the experts of the EULAR Sjogren's syndrome study group [10]. Furthermore, a more accurate evaluation of the infiltration extent in the LSG biopsy might also be provided by the assessment of two novel histopathological parameters, such as the total area of the inflammatory infiltrate and the percentage of inflammatory infiltrates. These features may reflect more accurately the complexity of the inflammation in LSG (i.e., presence of ectopic lymphoid structures), supporting a better identification of disease activity and a more accurate stratification of pSS patients [29,30].

Another tool to improve the reliability of histological assessment is digital image analysis. Recently described by Lucchesi et al., a digital approach for calculating either the total salivary gland area or the fraction occupied by the inflammatory infiltrate and the FS is capable of providing reproducible readings with a far superior inter-observer agreement compared to a grid-based approach [31].

Moreover, it is known that the histological abnormalities of the LSG biopsy, e.g., higher inflammatory infiltration and FS, correlates with various clinical and laboratory characteristics that might reflect an higher disease activity or an increased lymphoma risk [30, 32–34]. These clinical and laboratory features are listed in Table 13.1.

Specifically, in a standard multiple regression model, a FS ≥ 3 was significantly and independently associated with an increased risk of lymphoma development [35], while the role of GC and LELs in lymphoma prediction is still debated [16, 19–22].

Table 13.1 Clinical and serological features associated with higher glandular inflammatory infiltration on LSG biopsy

Clinical features	Serological features
Salivary gland swelling[34]	SSA/SSB positivity [30, 32, 34]
Lymph node and spleen enlargement [30]	Rheumatoid factor positivity[32, 34]
Vasculitis[30]	ANA positivity [30, 32, 34]
Keratoconjunctivitis sicca[32]	Leukopenia [30, 34]
Raynaud's phenomenon[30]	C4 hypocomplementemia[34]
	Hypergammaglobulinemia [32, 34]
	Circulating monoclonal component [34]

The correlation between LSG biopsy characteristics and functional variables (i.e., stimulated and unstimulated salivary flow rate) has also been analyzed [36, 37].

Currently, many clinical trials have evaluated histological changes on tissue samples after treatment (e.g., rituximab [38], abatacept [39], and belimumab [40]). However, the use of LSG biopsy as a predictive tool of treatment response deserves further evaluation, particularly if related to therapeutic agents that could have a significant positive effect on pSS clinical management.

Conclusion

Presently, LSG biopsy is the key point for the diagnosis and classification of pSS. Nevertheless, major obstacles remain, including the strict requirement of technical and interpretative procedures to be observed; thus, further standardization is needed. Importantly, LSG biopsy offers a unique opportunity to investigate pSS biology directly in the affected tissue, as well as to explore biomarkers, predictors of disease activity and lymphoma development.

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