Change in Final Diagnosis on Second Evaluation of Labial Minor Salivary Gland Biopsies

FREDERICK B. VIVINO, INDIRA GALA, and GEORGE A. HERMANN

ABSTRACT. ***Objective***. We evaluated the diagnostic accuracy of labial salivary gland specimens from a group of patients with symptoms or signs of dry mouth and/or dry eyes referred for assessment of possible Sjögren's syndrome (SS).

Methods. Fifty-eight individuals (52 women, 6 men; median age 54.5 yrs, range 19–90) had previously undergone one (n = 58) or 2 (n = 2) labial salivary gland biopsies, serologic studies, and objective tests for dry eyes and/or dry mouth to diagnose possible SS. Patients were referred to our institution for a second opinion regarding diagnosis and/or management of SS. All biopsy specimens underwent blinded review to measure aggregate glandular area, identify lymphocytic foci, and calculate focus scores that might verify the submitted diagnoses. Results were classified according to accepted histologic criteria: chronic sialadenitis, focal lymphocytic sialadenitis, indeterminate, insufficient tissue for diagnosis, and within normal limits. Institutional sources of submitted diagnoses included university hospitals (n = 26), university affiliates (n = 9), community hospitals (n = 18), commercial laboratories (n = 6), and a governmental agency (n = 1).

Results. Upon reexamination, 32 of 60 accessions (53%) sustained a revision of the initial diagnosis. Application of the focus scoring system combined with clinical features to reveal 12 hitherto undocumented cases of SS and refuted the diagnosis of SS in 8 instances. The principal reason for inaccurate initial interpretation was failure to apply the focus scoring system in 58 of 60 instances. Median diagnostic delay for the 12 SS cases was 302 days (range 55–2821).

Conclusion. It is possible that widespread cross-institutional failure to apply the focus scoring system in the interpretation of labial salivary gland biopsies may delay the recognition and/or treatment of SS. (J Rheumatol 2002;29:938–44)

Key Indexing Terms:
SALIVARY GLAND BIOPSY SJÖGREN'S SYNDROME

Despite limitations, labial salivary gland (LSG) biopsy constitutes an integral component of the several diagnostic algorithms proposed during the last 30 years to identify and classify Sjögren's syndrome (SS), an autoimmune exocrinopathy with clinical features of keratoconjunctivitis sicca, serologic abnormalities, and xerostomia[1-6]. Its diagnostic sensitivity varies from 70 to 83%, although specificity is uncertain, since characteristic lymphocytic infiltrates are reported in non-SS disorders[7,8].

Expert histologic diagnosis currently employs a semiquantitative focus score in the evaluation of focal accumulations of lymphocytes in LSG biopsies. This grading system, based on initial observations of Waterhouse and Doniach 35 years ago, has undergone continued refinement[8-12]. Recent investigation suggests that a focus score > 1.0/4 mm^2 correlates best with keratoconjunctivitis sicca, a characteristic feature of this disease[8]. Although today's research explores such issues as the Fas-Fas ligand system, apoptosis, and glucosamine binding, the focus score remains the most widely accepted and helpful tool in the histologic diagnosis of the salivary component of SS[13,14].

Published descriptions of LSG biopsy performance hail chiefly from institutions whose rheumatology, ear-nose-throat, ophthalmology, or dental medicine departments support interdisciplinary dry mouth/dry eye clinics. Histologic criteria in these centers of excellence may not match those of the general pathology community, where LSG material is often sparse, even at large centers with substantial surgical pathology caseloads.

We reexamined a cohort of LSG biopsies from institutions that largely lacked xerostomia clinics.

MATERIALS AND METHODS

Case population. Between February 1994 and December 2000 we reviewed 60 biopsies from 52 women and 6 men with a median age of 54.5 yrs (range 19–90). Two patients underwent repeat LSG biopsy within the 7 year interval. Patients had received their diagnoses classifying them into SS-consistent and SS-nonconsistent subpopulations up to 3880 days previously. The consulting rheumatologist requested a second review because of (1) diagnostic uncertainty in these cases' histologic descriptions or conclusions, (2) absence of marker autoantibodies, or (3) lack of objective

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evidence of dry eyes and/or dry mouth**.** The need to document autoimmunity in patients with sicca symptoms who lacked major serologic abnormalities was the most common stimulus for review of available material. No patients were excluded from analysis, even those with insufficient tissue according to morphometric criteria, because prior to the formal study one patient, after insufficient tissue for diagnosis was apparent, had undergone a second biopsy, which was consistent with SS. Institutional sources of LSG biopsies were split roughly 60:40 between non-university and university sites in eastern Pennsylvania and in New Jersey.

Clinical evaluation and diagnosis. We based our working diagnostic standards (Table 1) exclusively on modified European Community criteria[15]. Clinical evaluation included a complete history and physical examination, whole mouth unstimulated sialometry, $^{99m}TcO^-_4$ salivary scintigraphy, anesthetized Schirmer's test, Rose Bengal and fluorescein corneal staining, fluorescein tear breakup time, plus blood studies consisting of antinuclear antibodies, rheumatoid factor, anti-SSA/Ro, anti-SSB/La, serum protein electrophoresis, serum ß$_2$ microglobulin, and erythrocyte sedimentation rate by standard assays. Table 2 summarizes the clinical and laboratory features of the study group.

Table 1. Modified European community diagnostic criteria for Sjögren's syndrome.

Diagnosis of primary SS: any 4 of the following 6 criteria are met, including No. 5 or 6.
1. Oral symptoms (any 1)
Daily dry mouth > 3 months
Need of liquids for swallowing
Swollen salivary glands
2. Ocular symptoms (any 1)
Daily dry eyes > 3 months
Artificial tear use > 3×/day
Foreign body sensation
3. Oral signs (any 1)
Unstimulated salivary flow ≤ 0.1 ml/min
Abnormal salivary scintigraphy
Abnormal sialography
4. Ocular signs (any 1)
Schirmer's test ≤ 5 mm/5 min
Vital dye staining
Fluorescein tear breakup time < 10 s
5. Autoantibodies (any 1)
Positive anti-SSA/Ro or anti-SSB/La
ANA ≥ 1:160 or RF ≥ 1:160 (other causes excluded)
6.Histology
Labial minor salivary gland biopsy focus score > 1.0/4 mm^2

ANA: Antinuclear antibodies; RF: rheumatoid factor.

Histopathologic reevaluation. One or 2 pathologists examined all sections, strictly applying current diagnostic criteria[8]. Reevaluation employed a template that addressed the following features: number of glands, number of levels, number of slides, adequacy of fixation, staining and sectioning, aggregate glandular area, degree of interstitial fat and plasma cell infiltration (0–3+), estimated percentage of abnormal fibrotic area, condition of microvasculature, degree of acinar depletion and/or duct dilation (0–3+), gross focus number, focus score, diagnosis, and comment. All reviews proceeded without knowledge of the patient's clinical data or working clinical diagnosis and without knowledge of the initial histologic interpretation. The age and sex of the patients were known, however.

We defined gross lymphocytic foci as the presence of dense aggregates of 50 or more lymphocytes in perivascular or periductal locations, containing no more than 10% plasma cells, and located adjacent to normal-appearing acini in gland lobules with little or no duct dilation or fibrosis. If foci were present, a score > 1.0/4 mm^2 of parenchyma signaled focal lymphocytic sialadenitis (FLS) characteristic of the salivary component of SS (Figure 1). A focus score of 1.0 was indeterminate (IND), and focus scores < 1.0/4 mm^2 were not considered characteristic of SS. Focus scores of 10 or higher often became confluent, so we arbitrarily assigned a score of 12 to any specimen with this degree of lymphocytic infiltration. Scattered lymphoplasmacytic infiltrates associated with or without diffuse acinar atrophy, variable ductal dilatation, and interstitial fibrosis with focus

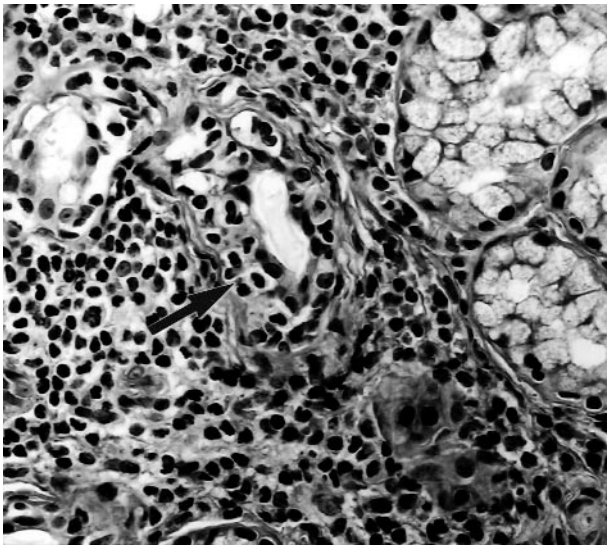

Figure 1. Focal lymphocytic sialadenitis. Intralobular duct surrounded by many lymphocytes. Arrow highlights lymphocytic infiltration of ductal epithelial cells. Adjacent acini at upper right are largely spared. Hematoxylin and eosin stain. Original magnification ×400.

Table 2. Subjective and objective feature of patient sample (n = 58)*

Subjective				Objective					
Oral		Ocular		Oral		Ocular		Serologic/other	
Symptom	Prevalence	Symptom	Prevalence	Abnormality	Prevalence	Abnormality	Prevalence	Abnormality	Prevalence
Dry mouth > 3 mo	51/58	Dry eyes > 3 mo	38/58	+ Salivary scintigraphy	28/45	Schirmer's ≤ 5 mm/5 min	17/46	ANA or RF ≥ 1:160	13/53
Salivary gland swelling	14/58	Foreign body sensation	9/58	Salivary flow rate < 0.1 ml/min	15/49	+Vital dye staining	20/42	+ SSA/Ro or SSB/La	5/53
Liquids for swallowing	17/58	Tear use > 3×/day	1/58			↓ Tear breakup time	18/39	↑ Serum β_2 microglobulin	6/44
								ESR > 50 mm/h	5/45

* Diagnostic studies incomplete in 20 instances. ANA: Antinuclear antibodies; RF: rheumatiod factor; ESR: erythrocyte sedimentation rate.

score < 1.0/4 mm^2 defined chronic sialadenitis (CS) (Figure 2). Biopsy material showing both FLS and CS was classified as FLS. Lobules showing extensive loss of parenchyma and diffuse fibrosis, a common sequel to severe or protracted chronic inflammation, were excluded from diagnostic evaluation (Figure 3). Biopsies with less than optimal (QNS) tissue areas (< 4.0 mm^2) or specimens containing < 4 glands were evaluated with a warning of the high risk of false negatives or sampling error. The remaining diagnoses were classified as within normal limits (WNL).

Sources of misdiagnosis. All cases sustaining a change in the submitted diagnosis were further examined in an effort to categorize the possible reason(s) for misdiagnoses. These consisted of: (1) no glandular area and/or no gross focus number and thus no focus score, (2) inaccurate measurement of surface area, (3) failure to recognize lymphocytic foci, (4) failure to recognize chronic sialadenitis, (5) failure to examine all tissue levels, and (6) failure to cite QNS/sampling error. Each such case was assigned one or more reasons for misdiagnosis.

Reproducibility of focus scores. Between July 1999 and December 2000, 2 pathologists (IG, GAH) independently measured the aggregate parenchymal area and tallied a gross focus number and focus score of 31 consecutive biopsies to evaluate interobserver precision and bias of estimation of these variables.

Statistical analysis. Because demographic, time delay, and other distributions were nonparametric, we used Wilcoxon signed rank tests that employed medians and ranges. P values ≤ 0.05 were considered significant. Least-squares regression analysis was used to evaluate the reproducibility and bias of tissue area, gross foci, and focus score estimates. Tissue area measurements were also graphically depicted in a scatter plot. Descriptive and analytic statistics were performed using NCSS 2000 software (Kaysville, UT, USA).

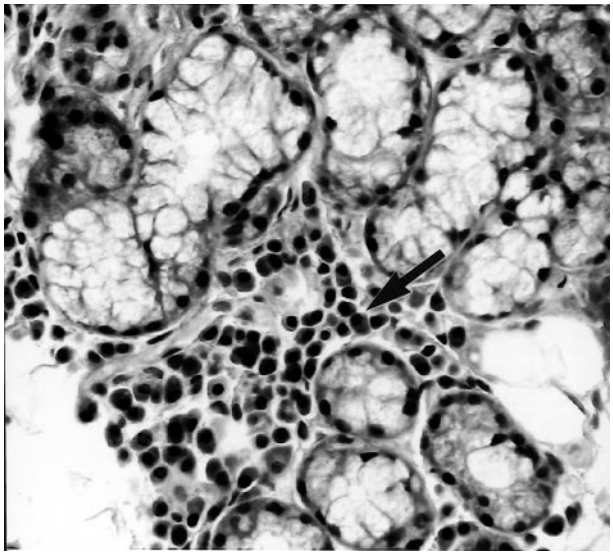

Figure 2. Chronic sialadenitis. Arrow identifies multiple interstitial plasmacytes surrounding normal acini. Hematoxylin and eosin stain. Original magnification ×400.

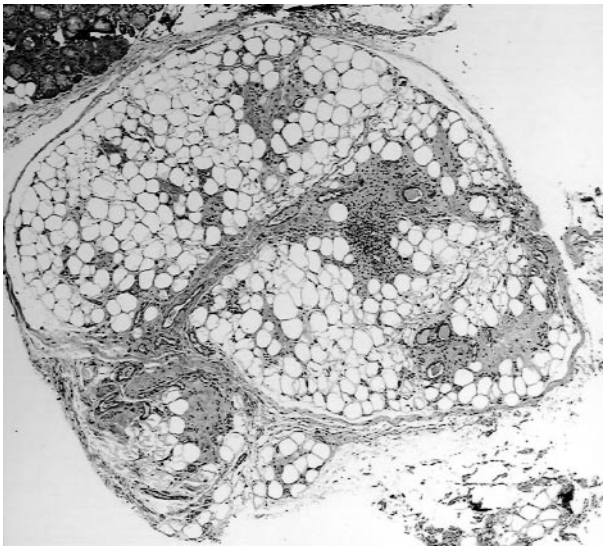

Figure 3. Minor salivary gland showing extensive fatty infiltration, parenchymal loss, and fibrotic foci that disqualify it from histologic evaluation. Hematoxylin and eosin stain. Original magnification ×40.

RESULTS

Diagnostic performance. Table 3 provides an overview of the technical and diagnostic aspects of cases, stratified into SS and non-SS groups. As expected, the focus scores of SS cases significantly exceeded their non-SS counterparts ($Z = 6.3$, $p << 0.001$). However, there was no statistical difference between the glandular areas ($Z = –0.45$, $p = 0.66$), although SS biopsies tended to provide slightly less tissue. Section levels per case in SS and non-SS groups did not differ significantly ($Z = 1.09$, $p = 0.28$). Additionally, there was no correlation between patient age and gross focus number or focus score, either in FLS-positive or FLS-negative cases. Table 4 compares submitting institutions' initial results with those of the review and lists days of diagnostic delay in the SS patients. Upon reexamination, 32 of 60 biopsies (53%) underwent diagnostic revision, resulting in a distributional shift from 29 chronic sialadenitis (CS), 19 focal lymphocytic sialadenitis (FLS), one indeterminate (IND), 4 miscellaneous, one less than optimal (QNS), and 6 within normal limits (WNL) to 24 CS, 25 FLS, 2 IND, 7 QNS, and 2 WNL. University departments, university affiliates, community hospitals, commercial laboratories, and a governmental agency had revision rates of 42, 67, 61, 67, and 0%, respectively (in a single case there was insufficient material for valid evaluation). Application of the focus score system uncovered 12 cases of FLS (14 LSG biopsies) and

Table 3. Technical and diagnostic variables of labial salivary gland biopsies, excluding indeterminate and insufficient diagnostic material categories.

Variable	Sample Population		
	Sjögren's, n = 25 Median (range)	Non-Sjögren's, n = 26 Median (range)	Total, n = 51 Median (range)
Slides/case	2 (1–4)	1 (1–3)	1 (1–4)
Glands/case	4 (1–7)	4 (2–7)	4 (1–7)
Levels/case	7 (2–28)	4 (2–25)	6 (2–28)
Glandular area, mm^2	8.6 (1.5–24.9)	9.5 (2.5–30.2)	9.2 (1.5–30.2)
Gross focus number	6 (2–28)	1 (0–6)	2 (0–28)
Focus score	2.8 (1.2–12)	0.4 (0–0.9)	0.9 (0–12.0)

Table 4. Change in diagnosis on second evaluation of labial salivary gland biopsies.

Case	Type of Institution	Submitted Diagnosis	Second Evaluation	Change?	Diagnostic Delay, SS, days
1	University	FLS	FLS	N	—
2	University	FLS	FLS	N	—
3	University	CS	CS	N	—
4	University	CS	FLS	Y	517
5	University	CS	CS	N	—
6	University	CS	FLS	Y	2007
7	University	CS	CS	N	—
8	University	WNL	CS	Y	—
9	University	FLS	FLS	N	—
10	University	FLS	CS	Y	—
11	University	FLS	FLS	N	—
12	University	CS	CS	N	—
13	University	FLS	CS	Y	—
14	University	WNL	IND	Y	—
15	University	FLS	FLS	N	—
16	University	CS	CS	N	—
17	University	FLS	CS	Y	—
18	University	CS	CS	N	—
19	University	MSC	CS	Y	—
20	University	CS	CS	N	—
21	University	CS	CS	N	—
22	University	FLS	CS	Y	—
23	University	CS	CS	N	—
24	University	CS	QNS	Y	—
25	University	CS	FLS	Y	81
26	University	CS	CS	N	—
27	University Affiliate	FLS	FLS	N	—
28	University Affiliate	CS	FLS	Y	264
29	University Affiliate	IND	QNS	Y	—
30	University Affiliate	FLS	FLS	N	—
31	University Affiliate	CS	FLS	Y	2394
32	University Affiliate	CS	FLS	Y	2821
33	University Affiliate	MCS	QNS	Y	—
34	University Affiliate	CS	CS	N	—
35	University Affiliate	WNL	CS	Y	—
36	Community	FLS	FLS	N	—
37	Community	FLS	IND	Y	—
38	Community	CS	FLS	Y	512
39	Community	FLS	FLS	N	—
40	Community	FLS	CS	Y	—
41	Community	CS	CS	N	—
42	Community	WNL	QNS	Y	—
43	Community	CS	FLS	Y	339
44	Community	WNL	WNL	N	—
45	Community	WNL	CS	Y	—
46	Community	CS	FLS	Y	207
47	Community	CS	CS	N	—
48	Community	QNS	QNS	N	—
49	Community	CS	FLS	Y	113
50	Community	CS	FLS	Y	117
51	Community	CS	FLS	Y	55
52	Community	MSC	FLS	Y	1592
53	Community	FLS	FLS	N	—
54	Commercial	CS	FLS	Y	55
55	Commercial	CS	CS	N	—
56	Commercial	FLS	FLS	N	—
57	Commercial	FLS	QNS	Y	—
58	Commercial	MSC	WNL	Y	—
59	Commercial`	FLS	QNS	Y	—
60	Governmental	CS	CS	N	—

CS: Chronic sialadenitis; FLS: Focal lymphocytic sialadenitis; IND: indeterminate; MSC: Miscellaneous (mucocele, squamous metaplasia on noncaseating granuloma); QNS: Insufficient for diagnosis; SS: Sjögren's syndrome; WNL: Within normal limits.

failed to confirm 8 others. As to SS, university departments had 4 false positive (FP) and 3 false negative (FN) diagnoses. Analogous totals for university affiliates, community hospitals, and commercial laboratories were 0 FP and 3 FN, one FP and 7 FN, and 2 FP and one FN, respectively. Should a histologic diagnosis of FLS have been needed to expand the preexisting clinical and laboratory database to confirm SS, the median diagnostic delay in such instances was 302 days with a range of 55 to 2821 days.

Sources of misdiagnosis. Fifty-eight of 60 submitted diagnoses were unaccompanied by a focus score. One university department and one university affiliate supported their diagnosis with a focus score. We confirmed both their surface area measurements and their diagnoses. Twenty-five additional deficiencies occurred — 12 misinterpretations of foci, 6 failures to diagnose chronic sialadenitis, 2 instances of failure to examine all sections, and 5 failures to cite QNS/sampling error. The 11 FN diagnoses in 34 non-university settings exceeded the 3 FN of 26 university departments, but the formers' 3 FP did not significantly differ from the latters' 4 FP. Since each group applied the focus scoring system in only one instance, it is not possible to determine the role of the focus score in this apparent difference. Exclusion of the 2 FP produced by commercial laboratories causes speculation that non-university hospital departments simply operate at more stringent subjective decision points on receiver operating characteristic curves similar in accuracy to those of their university counterparts.

Reproducibility and bias of areas and focus scores. Figure 4 plots the regression of the paired-observer glandular area estimates. Paired median areas were 8.0 and 8.5 mm^2, not significantly different ($Z = 0.55$, $p = 0.58$). Gross foci medians were 1.0 versus 2.0 ($Z = 1.7$, $p = 0.09$) and focus score medians were 0.9 versus 0.6 ($Z = 1.3$, $p = 0.2$). The regression equations of gross foci and of the focus scores were $Y = 0.77 \times +0.89$ ($n = 31$, $r = 0.95$) and $Y = 0.95 \times +0.17$ ($n = 31$, $r = 0.97$), respectively. Thus, reproducibility of aggregate parenchymal area, gross foci, and focus score assessment was high, with interobserver bias minimally noticeable in the gross focus score regression.

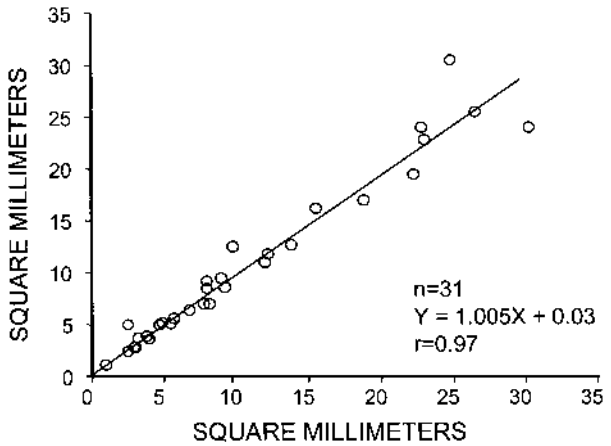

Figure 4. Correlation of independent paired measurements of glandular tissue area in labial salivary gland biopsies using a calibrated eyepiece graticle. Observer A, *x*-axis; observer B, *y*-axis.

DISCUSSION

Well defined and generally accepted histopathologic criteria for the diagnosis of FLS exist[8,9,11]. Our findings suggest they are not widely applied in general pathology practice. Over one-half of overall LSG diagnoses were revised, producing 22 FP or FN test results in the diagnosis of SS, an error rate of 37%. Median diagnostic delay exceeded 0.8 years in the SS patients.

Although several sources of error accounted for the high revision rate, the predominant deficiency involved a failure to employ the focus score system. Focus scores supported only 2 of 60 initial diagnoses. Reasons could include lack of awareness of the focus score system or unavailability of equipment for tissue measurement.

The diagnosis of primary or secondary SS can be a complex process that requires objective and subjective clinical evaluation with serologic and histopathologic data. Several criteria sets that assign variable weights to oral, ocular, and serologic components of this illness have evolved[1,2,4-6,15]. All systems, however, acknowledge that LSG biopsy contributes to diagnostic clarification, even though in many clinical settings it alone is neither necessary nor sufficient for diagnosis.

Minor salivary gland biopsy is not widely utilized in general rheumatologic practice and surgeons may have limited experience in technical and tissue adequacy requirements. Scarcity of case material also limits the general pathology community from rendering expert opinion. For instance, arguably the largest and most comprehensive published report of LSG biopsy, 618 cases, comes from a large academic institution with a highly active multidisciplinary dry mouth center[8]. Yet it took 20 years to accumulate the data, an average of 31 cases per year. From personal experience, it is not uncommon for pathology departments serving 500–600 bed medical centers to receive fewer than 3 LSG biopsies per year.

Valid calculation of focus score rests on 2 factors: (1) strict adherence to the definition of a lymphocytic focus and (2) reproducibility of area measurement. Current critical values of the focus score were established using a method in which precise, rather than highly accurate, area estimate is essential. However, little published data address its reproducibility. Our results suggest surprisingly good precision using inexpensive equipment, especially since the task requires a good deal of subjective tissue "carrying" due to irregularities of glandular contour (see Appendix). Thus, when combined with informed recognition of lymphocytic foci, area quantification makes the focus score a robust diagnostic vehicle. The enhanced accuracy and availability of

computer assisted morphometry may eventually supersede manual area measurement[16], but unless updated critical thresholds parallel technologic change, diagnostic performance may actually suffer.

Case selection bias may limit this retrospective study[17]. Clinical uncertainties prompt LSG biopsy, and ambiguity is likely to flourish among a cohort of patients perceived to need second opinions. Situations that elicited a slide review in this study included the need to document salivary gland involvement through demonstration of characteristic histologic changes when a diagnosis could not be achieved by other methods. It is likely that histologic data deemed inconsistent with a working diagnosis based on clinical history, physical examination, laboratory evaluation, and serologic results would be suspect prior to review. Thus the substantial diagnostic revision rate we report might not be applicable to biopsies in the general SS population. Nevertheless, an absence of focus scores in 97% of reviewed cases makes it likely that the system does not currently enjoy widespread utilization.

The question remains whether one is dealing with a phenomenon of the mid-Atlantic region, source of our samples, or whether it is nationally endemic. Similar discrepancies have been reported in routine pathologic review of other gastrointestinal material at Midwest institutions[18]. Two of our patients may anecdotally highlight hazards of diagnostic opinion unaided by standardized criteria. A biopsy with a 0.9 mm^2 glandular area was diagnosed as FLS. The material was less than optimal since a single lymphocytic focus, given this area, would have yielded a focus score of 4.4, clearly abnormal. Another patient with a focus score of 1.8 carried an initial diagnosis of mild chronic sialadenitis. Four years later clinical deterioration prompted a repeat biopsy that was diagnosed in the same institution as an organizing mucocele. The patient's focus score in the second biopsy had risen to 2.9.

In summary, slightly more than half of LSG biopsies reevaluated because of clinical-pathologic discordance in the mid-Atlantic region of the US failed to confirm the initial histologic diagnosis. Failure was found at community hospitals as well as university associated institutions, commercial laboratories, and university centers. Suspicion falls on non-application of the focus score system as the source of these discrepancies. Determination of whether such findings apply as well to the unreviewed national pool of LSG biopsies requires a larger and less biased sample. Rheumatologists and their patients facing the diagnostic uncertainties of SS would be well served by colleagues who understand and employ the focus score system.

ACKNOWLEDGMENT

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APPENDIX

Focus scoring. Detailed descriptions of the counting technique used for determining focus score are not widely distributed. The following guidelines (T.E. Daniels, written communication) are offered. Only lobules containing glandular parenchyma, excluding periglandular fibrous tissue, fat, voluntary muscle and large blood vessels, are counted. Multiple levels of tissue should be used to maximize the number of foci, glandular area, and technical quality of the material. The microscopist needs a 10 × 10 square eyepiece graticle, purchasable at any scientific optical instrument retailer, and a 2× objective. A stage micrometer, available in most pathology departments, calibrates graticle line segments into millimeters at any magnification. With a 2× objective and 10× eyepiece, each graticle cell segment measures roughly 0.5 mm. The area of each square is therefore 0.25 mm^2. The sum of 4 such squares is 1 mm^2. Count the total number of squares overlying each gland. Since glandular outlines are irregular and tissue often partially fills several cells, one must estimate the proportion of each square occupied by tissue and combine it with an analogous square to create a fully occupied, and thus countable, unit. Surprisingly, this process of "carrying" of tissue images detracts little from the precision of measurement. To determine the total glandular area in mm^2 divide the total number of tissue-occupied cells by the number of cells equaling 1 mm^2. To obtain foci/mm^2, count the number of lymphocytic foci in the specimen using previously described criteria, and divide by the total glandular area. To obtain the number of lymphocytic foci in 4 mm^2 of gland, the focus score, multiply the foci/mm^2 by 4. For example, in a section containing 5 minor salivary glands, the various glands occupy, respectively, 17, 20, 14, 26, and 16 cells, a total of 93. Using the calibration factor associated with the 2× objective, 4 squares/mm^2, the total glandular area is 93/4 = 23 mm^2. If there are 21 lymphocytic foci in the section, the focus score is (21/23) × 4 = 3.7/4 mm^2. If a 4× objective is used, about 16 boxes will comprise 1 mm^2. We have found that the majority of specimens are most conveniently counted with a 2× objective, although some smaller tissue samples may require 4×. In principle, use of higher power objectives is feasible, although seldom necessary.

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