Objective: To assess the evidence for *Borrelia burgdorferi* sensu lato infection in patients with lichen sclerosus by focus-floating microscopy.

Setting: Dermatology department of a university hospital.

Design: Tissue sections were stained with a polyclonal *B burgdorferi* antibody using standard histological equipment and then scanned simultaneously in 2 planes: horizontally in a serpentinelike pattern and vertically by focusing through the thickness of the section, ie, focus-floating microscopy. Part of the material was also investigated by *Borrelia*-specific polymerase chain reaction.

Patients: The study population comprised 61 cases of lichen sclerosus and 118 controls (60 negative controls and 68 positive controls).

Main Outcome Measure: The presence of *B burgdorferi* sensu lato within tissue specimens.

Results: Using focus-floating microscopy, we detected *Borrelia* species in 38 of 60 cases (63%) of lichen sclerosus and in 61 of 68 (90%) of positive controls of classic borreliosis, but *Borrelia* species were absent in all negative controls. *Borrelia* species were detected significantly more often in early inflammatory-rich (31 of 39 [80%]) than in late inflammatory-poor (7 of 21 [33.3%]) cases (P = .001). Polymerase chain reaction findings were positive in 25 of 68 positive controls (37%) and negative in all 11 cases of lichen sclerosus and all 15 negative controls.

Conclusions: Focus-floating microscopy is a reliable method to detect *Borrelia* species in tissue sections. The frequent detection of this microorganism, especially in early lichen sclerosus, points to a specific involvement of *B burgdorferi* or other similar strains in the development or as a trigger of this disease.

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Lichen sclerosus (LS), frequently reported in the dermatologic literature as lichen sclerosus et atrophicus, is a chronic inflammatory skin disease of unknown etiology that leads to substantial discomfort and morbidity. It commonly affects adult women in the genital-anal region ([Figure 1A](#)) but also occurs elsewhere.¹⁻⁴ Lichen sclerosus has clinical and histological similarities with morphea, and some investigators consider this entity a superficial variant of morphea, an opinion supported by its frequent coincidence with morphea.⁵⁻⁶ Lichen sclerosus shares similarities and common features with acrodermatitis chronica atrophicans (ACA), a chronic form of borreliosis, particularly histological findings such as an infiltrate of lymphocytes admixed with some plasma cells, an increase in fibrocytes and fibroblasts, and a diffuse dermal fibrosis to sclerosis ([Figure 1B and C](#)).⁷ These observations have led several investigators to consider the possibility of *Borrelia burgdorferi* sensu lato as a common etiologic factor for both diseases. Since the first proposal of *B burgdorferi* as a causative agent by Aberer and Stanek⁸ in 1987, conflicting results have been obtained by different studies using serological, immunohistochemical, culture, and polymerase chain reaction (PCR) approaches. *Borrelia* species have frequently been detected in Europe, but not in cases from the United States. Studies reporting a positive association between *B burgdorferi* infection and LS found evidence of the organism in 10% to 68% of cases; on the other hand, there are reports in which no positive cases could be identified ([Table 1](#)).⁹⁻²⁰

One main difficulty in assessing the association between LS and borreliosis is the

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challenge to reliably detect *B burgdorferi* in tissue specimens. These conflicting results at least in part reflect the difficulties of the various techniques used to document the participation of *Borrelia* species in the disease process. Therefore, serological techniques are unsatisfying, with false-negative (20%-80%) and false-positive (20%-50%) results in classic manifestations of borreliosis, such as erythema migrans (EM), borreliotic lymphocytoma (BL), and ACA. A negative serological test result does not exclude previous infection with *B burgdorferi*, and a positive result may represent an endemic background.21-23 Histological, histochemical, and immunohistochemical detection of microorganisms has turned out to be difficult, frequently unreliable, and almost always extremely time consuming.23-26 Cultures with specific media can detect *Borrelia* species in all clinical forms, but these techniques are not generally available and are unreliable, with less than 50% sensitivity for classic *Borrelia* infections. Therefore, negative culture findings may be attributable to the fastidiousness of the organism in the culture.26,27 The initial enthusiasm with molecular techniques gave way to a more realistic evaluation of these methods as it became clear that sensitivity varies (30%-90%) according to *Borrelia* strains, the material (fresh, frozen, or paraffin material), and the primers applied.26,28-33 In summary, all current detection methods seem to bear an inadequate sensitivity for the detection of *Borrelia* species, so even the classic cutaneous *Borrelia* infections remain a diagnosis based on circumstantial evidence combining clinicopathologic and laboratory information and response to therapy.

We recently developed a highly sensitive immunohistochemical procedure that proved to be more sensitive than PCR in the detection of *B burgdorferi sensu lato* in classic cutaneous borreliosis (98% vs 45%) and nearly equally specific (99% vs 100%).28 We named this procedure focus-floating microscopy (FFM). In cases in which abundant *B burgdorferi* were detected by FFM were usually positive by PCR and when fewer organisms were detected by FFM, specimens were more likely to be negative by PCR. Using this new technique, we tried to assess the evidence for infection with *B burgdorferi sensu lato* in patients with LS.

### METHODS

#### PATIENTS

We searched the files of the Dermatohistopathological Laboratory in Innsbruck, Austria, from the years 1989 through 2006, and retrieved 61 cases of LS. Diagnoses were well established by exact correlation with clinicopathological diagnosis for LS, including photographic documentation in many instances. Serological test results were present only for a minority of patients and were not usable because of the a priori high endemic background of positive *Borrelia* serological status in our geographic area.21,22

### Table 1. Results of Studies Investigating *Borrelia burgdorferi* in Patients With Lichen Sclerosis

<table>
<thead>
<tr>
<th>Source</th>
<th>Country</th>
<th>Method</th>
<th>Results, No. Pos/Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberer et al,1987</td>
<td>Austria</td>
<td>Immunoperoxidase</td>
<td>6/13</td>
</tr>
<tr>
<td>Aberer et al,1988</td>
<td>Austria</td>
<td>Immunoperoxidase</td>
<td>1/2</td>
</tr>
<tr>
<td>Ross et al,1990</td>
<td>Puerto Rico</td>
<td>Silver stain</td>
<td>10/21</td>
</tr>
<tr>
<td>Scherff et al,1993</td>
<td>Germany</td>
<td>PCR (flagellin)</td>
<td>6/6</td>
</tr>
<tr>
<td>Ranki et al,1994</td>
<td>Finland</td>
<td>PCR (OspA)</td>
<td>0/1</td>
</tr>
<tr>
<td>Dillon et al,1995</td>
<td>United States</td>
<td>PCR (flagellin)</td>
<td>0/10</td>
</tr>
<tr>
<td>De Vito et al,1996</td>
<td>United States</td>
<td>PCR (clone 2H1)</td>
<td>0/7</td>
</tr>
<tr>
<td>Fujiiwara et al,1997</td>
<td>Japan</td>
<td>PCR (16S RNA)</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>Germany</td>
<td>PCR (16S RNA)</td>
<td>1/10</td>
</tr>
<tr>
<td>Colome-Grimmer et al,1997</td>
<td>United States</td>
<td>PCR (flagellin)</td>
<td>0/10</td>
</tr>
<tr>
<td>Alonso-Llanazares et al,1997</td>
<td>Spain</td>
<td>PCR (OspA)</td>
<td>0/8</td>
</tr>
<tr>
<td>Aberer et al,1999</td>
<td>Austria</td>
<td>Culture</td>
<td>0/1</td>
</tr>
<tr>
<td>Ozkan et al,2000</td>
<td>Turkey</td>
<td>PCR (flagellin)</td>
<td>13/19</td>
</tr>
<tr>
<td>Breier et al,2001</td>
<td>Austria</td>
<td>Culture</td>
<td>6/12</td>
</tr>
<tr>
<td>Present study, 2008</td>
<td>Austria</td>
<td>PCR (23S RNA)</td>
<td>0/11</td>
</tr>
</tbody>
</table>

Abbreviations: FFM, focus-floating microscopy; PCR, polymerase chain reaction; Pos, positive.

*For the studies involving PCR, the amplified gene is shown in parenthesis.*
Sixty cases, mainly inflammatory skin lesions, served as negative controls, and 68 cases of PCR-controlled, clinically and histologically characteristic *Borrelia* infections (15 cases of EM, 23 cases of BL, and 34 cases of ACA) served as positive controls. Because our archival paraffin material had been fixed in inadequately buffered formalin until 2004, we could perform *Borrelia*-specific PCR in only 11 LS cases. In 10 of these cases, there was enough paraffin material left to further complete FFM.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Serial sections from paraffin-embedded, formalin-fixed tissue blocks were obtained for hematoxylin-eosin staining and immunohistochemical analysis as previously described.24 Briefly, we used a polyclonal rabbit antibody (Acris BP1002, derived from immunization with whole-cell *B. burgdorferi* preparations [strain B31, ATCC 35210; American Type Culture Collection, Manassas, Virginia] reacting with 83-kD and 41-kD flagellin, 32-kD OspB, and 31-kD OspA antigens and their fragments in Western blots, with cross-reaction to *Treponema pallidum*, *Borrelia hermsii*, and *Borrelia parkeri*) at a dilution of 1:2000, with an autoclave antigen retrieval time of 30 minutes in a sodium citrate buffer (pH 6.0-6.1) and an incubation time of 30 minutes at 37°C. Further steps followed the Ventana-KIT (Ventana Medical Systems, Munich, Germany) method as routinely used for immunohistochemical analysis in our laboratory with a biotinylated second antibody and a streptavidin-biotin horseradish peroxidase complex as a third layer. As a final reaction product, we used 3-amino-9-ethylcarbazole, whose bright red color proved superior to the brown color of diaminobenzidine. The counterstain was omitted to enable easier recognition of *Borrelia* species. We first examined all immunohistochemical stains for the presence of *Borrelia* species independently (K.E., T.G., and B.Z.), including LS cases and positive and negative controls. Absence of counterstains guaranteed that these sections were evaluated in a blinded fashion, with the investigator unaware of the pathological diagnosis. There was excellent interobserver reliability among the different investigators. In rare occasions of divergent evaluation, subtle presence of *Borrelia* species had been overseen by one or the other investigator. After evaluation for *Borrelia* species by FFM, we correlated the results with the hematoxylin-eosin stains. In the case of *Borrelia* species detection, serial sections allowed for the exact localization of microorganisms in relation to the disease process.

**FOCUS-FLOATING MICROSCOPY**

Focus-floating microscopy (Figure 2) is a modified immunohistochemical technique, which combines several strategies to detect minuscule organisms in tissue sections.24 Focus-
floating microscopy scans through the sections in 2 planes: horizontally in a serpentinelike pattern, as in routine cyto-
logic examination, and, simultaneously, vertically by focusing through the thickness of the cut (usually 3-4 µm) at an origi-
nal magnification of ×200 to ×400. This holographic ap-
proach allows for the detection of *B burgdorferi* (diameter of 0.2 µm, compared with 2.0 µm for collagen bundles), which pass through the section at various angles and accordingly may appear as undulated, comma- to dot-shaped forms. In addi-
tion, the omission of counterstaining as well as bright illumi-
nation of the scanning field proves to be helpful because the bright red color of the 3-aminophenylcarbazole–stained mi-
croorganisms best contrasts with the faint yellow color of un-
stained collagen bundles as well as other tissue structures.

**POLYMERASE CHAIN REACTION**

For molecular identification of *B burgdorferi*, DNA was prepared from paraffin-embedded tissue. After deparaffinization with xylene and ethanol and digestion with 0.6-mg proteinase K for 16 hours, the remaining DNA was purified by adsorption chroma-
tography (QIAamp DNA Mini Kit; QIAGEN GmbH, Hilden, Germany), and the concentration of the sample was adjusted to 10 
mg/L. Nested PCR was performed in volumes of 25 µL with 30-ng DNA, 100 pmol of each primer, 10mM Tris hydrochloride (pH 9.0), 50mM potassium chloride, 1.5mM magnesium chloride, 200mM of each deoxyribonucleotide triphosphate, and 1.5 U of *Taq* polymerase. The samples were subjected to the following condi-
tions: in a PTC 200 thermocycler (MJ Research Inc, Water-
town, Massachusetts), the first PCR was performed for 30 sec-
onds at 94°C, 30 seconds at 53°C, and 30 seconds at 72°C for 40 cycles; and the second PCR was performed for 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C for 45 cycles. For amplification, the following primers specific for the *B burgdorferi* 23S ribosomal RNA gene35 were used: for the first PCR, Bor-1: 5'-AGAAGTTGGGAGTGAAGA-3' and Bor-2: 5’-TAGTGTGCT-
TACCTCAT-TAA-3’; and for the second PCR, Bor-3: 5’-
GCGAAAGCGAGTCTTAAAAGG-3’; and Bor-4: 5’-
ACTAAAAATGAGCTGAACATTAAAT-3’. After separation on a 2% agarose gel (50 mA for 30 minutes) and staining with ethidium bromide, the PCR product of 219 base pairs was visualized un-
der UV light (302 nm).

**STATISTICAL ANALYSIS**

Data were statistically analyzed using SPSS statistical software (SPSS for Windows, version 12.0; SPSS Inc, Chicago, Illinois). Statistical comparisons were performed using the 2-tailed Pear-
son χ² test or the Fisher exact test when appropriate. *P < .05* was considered statistically significant.

**RESULTS**

The median age of the patients was 58 years (mean age, 
55.1 years; minimum age, 5 years; and maximum age, 
86 years), and 22 patients (36%) were male and 39 (64%) 
were female. Biopsy specimens were mainly obtained from 
the extremities, with a minimum age of 5 years and a 
maximum age of 86 years. Of the 60 LS cases, 38 were positive by FFM (63%). The presence of *Borrelia* species was similar in pure LS cases and in those associated with morphea (33 of 52 [64%] vs 5 of 8 [63%]). *Borrelia burgdorferi* sensu lato was detected signifi-
cantly more often in early inflammatory-rich (31 of 39 [80%]) than in late inflammatory-poor (7 of 21 [33%]) cases (P = .001). Similarly, an early inflammatory-rich case
of LS usually revealed more microorganisms than a late inflammatory-poor case. Statistically, all specimens of LS revealed significantly less spirochetes than cases of classic borreliosis (ie, EM, BL, and ACA) (89.7% vs 63.3%; \( P = .001 \)). This significance was lost when compared with early inflammatory-rich forms of LS (80% vs 90%; \( P = .16 \)). Focus-floating microscopy was much more sensitive to detect Borrelia species in controls compared with PCR (90% vs 37%; \( P < .001 \)). Yet, none of 11 LS specimens was positive by PCR, while 6 of 10 of these PCR-negative cases were positive by FFM, with 1 case not having material left to perform FFM after PCR.

The B. burgdorferi antibody showed no cross-reactions with other tissue structures. All 60 controls from well-defined mainly inflammatory disorders other than borreliosis remained negative. In our experience, silver techniques over the last decades never proved to be successful for the reliable detection of microorganisms in routine laboratory procedure and thus were not performed in this study.

**COMMENT**

The involvement of B. burgdorferi as a causative agent for LS was first proposed by Aberer and Stanek\(^8\) in 1987 and was subsequently further supported, at least in part, by several other studies (Table 1).\(^8,20\) A bacterial cause was further suggested because several cases of LS responded well to therapy with antibiotics, such as dirithromycin, penicillin, and ceftriaxone.\(^20,36,37\)

In the present study, we detected B. burgdorferi sensu lato in more than 60% of all LS cases, with a significantly higher percentage (\( P = .001 \)) in early (80%) than in late (33%) LS, while it made no difference whether LS was associated with morphea. This might reflect intentional or coincidental antibiotic exposure in longer-term cases and/or the natural course of disease, with repression of the microorganism by the immune system. The negative detection rate of Borrelia DNA by PCR in our study, with no positive case in 11 tested, indicates the problematic role of this technique to reliably detect Borrelia species in tissue specimens.

The low number of microorganisms beyond the detection threshold\(^38\) could be one explanation for the inconsistent results in PCR studies (Table 1). Other explanations include old stage of disease, wrong biopsy site (eg, from negative fibrosclerotic parts), or wrong fixation of tissue specimens leading to DNA cross-linking (eg, with inadequately buffered formalin). Furthermore, ex-

\[ \text{Figure 3. Four different cases of late lichen sclerosus, in which single spirochetes were found using focus-floating microscopy. The photographs show 4 different morphological appearances of Borrelia species: elbow form (A), bacilli form (B), undulated form (C), and bacilli and dot-shaped (D). (Immunohistochemical analysis for Borrelia burgdorferi, Acris BP1002 [polyclonal rabbit antibody], no counterstain, original magnification \( \times 1000 \).)} \]
cept for the studies by Ranki et al\(^{12}\) and Dillon et al,\(^{13}\) other PCR studies with negative findings are lacking appropriate positive controls in terms of detection of *Borrelia* DNA in tissue specimens from classic borreliosis such as EM, BL, and ACA. Thus, the reliability of the DNA extraction method for small DNA amounts or the PCR technique used in these studies remains somewhat debatable.\(^{14,16}\)

There is still another explanation for negative PCR results: *B. burgdorferi* sensu lato includes *B. burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii*, but newer *Borrelia* species have been identified. The pathogenic significance of these species, such as *Borrelia valaisiana, B. hermsii, Borrelia turicatae, Borrelia duttonii, B. parkeri*, and most recently *Borrelia spielmanii* is not yet fully answered. While *B. burgdorferi* sensu stricto, to the best of our knowledge, is the only cause of Lyme disease in the United States, *B. afzelii, B. garinii,* and probably *B. valaisiana* also cause Lyme disease in Europe and Asia. Relapsing fever borreliosis caused by *B. hermsii, B. turicatae,* and *B. duttonii* and EM caused by *B. spielmanii* have been described.\(^{19,40}\) The study by van Dam et al\(^{11}\) suggests that different *B. burgdorferi* genotypes have different pathogenic potentials. This is well documented for the classic *Borrelia* manifestations; for example, ACA rarely occurs in the United States but is commonly seen in Europe where *B. afzelii* and *B. garinii* are more prevalent.\(^{42}\) Maybe subspecies variations dictate the clinical manifestations that follow infections, with only certain strains possessing the characteristics required to initiate the development of LS.\(^{15}\) Thus, another explanation for the moderate results by PCR might be that these techniques use primers highly specific for known human pathogenetic strains, whereas FFM uses an immunohistochemical approach involving a less specific polyclonal antibody that probably detects more different *Borrelia* species. *Borrelia* species have not been implicated as a cause of LS in the United States, and we did not have the opportunity to examine cases from other patient populations. Therefore, it remains to be seen if the use of this technique would reveal cases of *Borrelia*-associated LS in the United States.

In any case, detection of spirochetes in pure LS and LS associated with morphea seems to be a common denominator, which indicates the nosologic relationship of these skin disorders.\(^{8,13}\) Moreover, the infectious hypothesis with spirochetes helps to explain the most common stereotypical presentation of LS, namely in the genital-anal area. Subclinical dissemination with the spread of

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**Figure 4.** Series of magnifications (original magnification ×100 [A], ×200 [B], ×400 [C], and ×1000 [D]) from the right lower part of the biopsy specimen (arrow indicates corresponding structures) allow for the identification of 3 clusters of long and partly undulated, plump, granular, or delicate microorganisms. Note the faint red, diffuse staining of subepidermal fibrosis left of the infundibula of 2 hair follicles, while clusters of spirochetes are at the periphery of the inflammatory process in the mid dermis. (Immunohistochemical analysis for *Borrelia burgdorferi*, Acris BP1002 [polyclonal rabbit antibody], no counterstain.)
Borrelia to kidneys and urine occurs in early Borrelia infection.34,47 Favoring by the moist and frequently traumatized conditions of genitalia, this might allow a superficial Borrelia infection in the perigenital region (ie, LS). This could explain the frequent occurrence of the disease in the perigenital area and why other mucous membranes such as the oral or endonasal mucosa and conjunctiva are practically never affected. The lower level of microorganisms in late LS not only indicates that the disease is the consequence of the infectious agent but also reflects the challenge for the immune system and/or a compromised immune reaction in the patients themselves, in whom Borrelia antigens might trigger a subsequent autoimmune reaction in genetically predisposed individuals via molecular mimicry.43 Thus, thyroid autoantibodies have been described in 36% of patients with LS.45 Borrelia burgdorferi has been proposed as an environmental trigger of autoimmune thyroiditis through amino acid sequence homologies between proteins of B burgdorferi and all thyroid autoantigens (eg, human thyroid-stimulating receptor, human thyroglobulin, human thyroperoxidase, and human sodium iodide symporter) or segments thereof.46,47 The induction of autoimmunity also might explain why not all patients benefit from antibiotic therapy and makes an early antimicrobial treatment reasonable.

In conclusion, FFM is a reliable method to detect Borrelia species in tissue sections, and the frequent detection of this microorganism, especially in early LS, points to a specific involvement of B burgdorferi or other similar strains in the development or as a trigger of LS.

Table 2. Detection of Borrelia species by FFM and PCR in Lichen Sclerosus (LS) Cases and Controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>FFM</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification of LS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS, “pure”</td>
<td>33/52 (64)</td>
<td>0/5</td>
</tr>
<tr>
<td>LS with morphea</td>
<td>5/8 (63)</td>
<td>0/6</td>
</tr>
<tr>
<td>Stage (activity) of LS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory rich, “early”</td>
<td>31/39 (80)</td>
<td>0/7</td>
</tr>
<tr>
<td>Inflammatory poor, “late”</td>
<td>7/21 (33)</td>
<td>0/4</td>
</tr>
<tr>
<td>Total</td>
<td>38/60 (63)</td>
<td>0/11</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0/60</td>
<td>0/15</td>
</tr>
<tr>
<td>Positive</td>
<td>61/68 (90)</td>
<td>25/68 (36.8)</td>
</tr>
</tbody>
</table>

Abbreviations: FFM, focus-floating microscopy; PCR, polymerase chain reaction.

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Author Contributions: Dr Eisenheld had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Eisenheld, Kutzner, and Zelger. Acquisition of data: Eisenheld, Grabner, Kutzner, and Zelger. Analysis and interpretation of data: Eisenheld and Zelger. Drafting of the manuscript: Eisenheld and Zelger. Critical revision of the manuscript for important intellectual content: Grabner, Kutzner, and Zelger. Statistical analysis: Eisenheld. Administrative, technical, and material support: Kutzner and Zelger. Study supervision: Zelger.

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