Exploring reflectance confocal microscopy as a non-invasive diagnostic tool for genital lichen sclerosus

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Abstract. The diagnosis of genital lichen sclerosus (LS) is often confirmed by obtaining a skin biopsy, which can lead to unwanted complications and is uncomfortable in the sensitive genital area. Thus, there is a need of finding novel, non-invasive techniques that can rapidly and accurately diagnose LS. The present study investigated the potential for reflectance confocal microscopy (RCM) to diagnose LS compared with healthy penile skin and other common penile skin disorders in males. A total of 30 male patients, including patients with LS, nonspecific balanoposthitis, plasma cell balanitis and psoriasis, and healthy individuals were included and were subject to non-invasive RCM investigation. Prominent fiber-like structures, representing hyaline sclerosis, were observed in the RCM images for almost half of the patients. Differences between healthy penile skin and LS were confirmed by identifying the edged papillae on healthy skin and their absence or obscureness in patients with LS. Notably, RCM could detect the atypical honeycomb pattern referring to dysplasia in 1 patient with LS with penile intraepithelial neoplasia. In conclusion, the present study demonstrated that RCM can detect sclerosis in penile LS. RCM can potentially become a valuable tool for monitoring patients with LS for dysplasia providing a useful non-invasive diagnostic tool for genital disorders.

Introduction

Lichen sclerosus (LS) is a chronic inflammatory skin disease with a predilection for the anogenital area. The estimated prevalence of the disease is 1:300-1:1,000 and it is primarily seen in postmenopausal women, although men and children also can be affected (1). In men, LS occurs mainly between the ages of 30 to 50 years (2,3). LS is presented clinically with hypopigmentated areas, petechiae, and in males also with preputial and meatal constriction (4). In females, LS has been associated with an increased risk of vulvar squamous cell carcinoma (SCC), estimated at 2.6-6.7% (5). Also, in males an increased rate of SCC has been shown with a prevalence of 1-6% (3,4,6,7).

The diagnosis of LS is often based on the aforementioned, typical clinical criteria. Dermoscopy can give additional information and thereby assisting the diagnosis (8). Nevertheless, in most patients, a skin biopsy is required to confirm the diagnosis of LS and, in some cases, to rule out SCC. The typical histological features of LS are a thinned epidermis, a dermal hyaline sclerosis and below this a band-like chronic inflammation (9). The genital LS can also lack epidermal atrophy and in some cases show spongiosis. The use of invasive biopsies in the genital area is not always uncomplicated. This sensitive area has a dense network of blood vessels that can cause bleeding and aesthetic problems, like scars, can be seen after a skin biopsy. Furthermore, the skin biopsy is associated with diagnosis delay and laboratory costs. Therefore, there is a need for finding a fast, accurate, and non-invasive diagnostic procedure for LS.

Non-invasive imaging techniques for medical diagnostics have evolved over the last decade. Among optical microscopy techniques, laser scanning microscopy has been particularly promising because it allows for a three-dimensional, non-invasive visualization of biological tissue with high resolution (10,11).

Reflectance confocal microscopy (RCM) is a well-established laser scanning microscopy technique for imaging skin *in vivo*. RCM achieves contrast by utilizing the inherent refractive index properties of various cellular microstructures. Commercially available RCM devices create images with a resolution comparable to histological examination (12). RCM is an emerging tool for skin cancer diagnostics (13,14). Moreover, several studies have shown that RCM can be used to diagnose psoriasiform and interface dermatitis (15-17). The epidermal layer of the prepuce and glans which is approximately 140 μm (18,19) could theoretically be appropriate for investigation with RCM, which has an image depth of approximately 150-200 μm, reaching the papillary dermis.

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There are preliminary reports that RCM has been used as a complementary diagnostic and monitoring tool for LS (20,21). Nevertheless, these studies contain a small number of patients and they lack comparison to the healthy skin. More studies are needed to confirm existing data. Multiphoton microscopy (MPM) is a related technology to RCM (22). However, its translation into the clinics has so far not proceeded to the same extent as RCM.

Thus, in this descriptive study we aimed to investigate the potential for laser scanning microscopy, and RCM in particular, as a diagnostic tool for LS in comparison to normal penile skin and other penile inflammatory disorders. Furthermore, we used MPM *ex vivo* in one skin biopsy from one LS patient to compare the findings to those of the RCM. In addition to assessing the diagnostic potential of the approach, the recruited patients were asked to assess their experience of diagnostic procedure experience.

Materials and methods

Participants in the study. All the participants signed an informed consent form. The study was approved by the regional ethical review board of Gothenburg (Dnr 415-17), and institutional rules for the clinical investigation of human subjects were followed. The inclusion criteria were ≥18 years of age, histopathologically confirmed LS. As controls, clinically diagnosed nonspecific balanoposthitis, plasma cell balanitis, psoriasis and healthy individuals were included. The participants were not allowed to apply any topical treatment on the genital area 14 days prior to the inclusion in the study. Patients were recruited at the Department of Dermatology and Venereology, at the Sahlgrenska University Hospital, in Gothenburg, Sweden, from 2018 to 2020. In total, 30 male patients were included of which 17 patients were diagnosed with LS, five patients with nonspecific balanoposthitis, three with plasma cell balanitis, one patient with psoriasis and four were healthy individuals. The date of the obtained skin biopsy confirming the LS diagnosis varied from eight years prior to the inclusion up to the same day of the inclusion. Asymptomatic patients visiting the clinic to exclude sexually transmitted diseases, and patients evaluated for extragenital skin disorders, were recruited as controls with healthy penile skin. All patients were subject to RCM investigation, as described below. The prepuce was investigated with RCM in all cases. All the participants were asked if they had oral LS and all of them denied it. The oral cavity was not examined since oral LS very rarely occurs in this location. In addition, the patients diagnosed with LS answered a questionnaire that contained inquiries related to LS, circumcision, treatment, and experiences from the biopsy procedure.

Reflectance confocal laser scanning microscopy. All patients were examined using an *in vivo* RCM (VivaScope 1500™, MAVIG GmbH,), using an adopted protocol based on an established clinical routine for dermatological investigations. Oil was applied to an adhesive window attached to a stainless-steel tissue ring. The window was placed onto the affected or healthy penile skin. Ultrasound gel was applied to the center of the adhesive window. Then, the laser tube of the RCM was affixed to the tissue ring. To be oriented during imaging, a dermoscopic image was obtained with the VivaCam, which is incorporated into the VivaScope system. A standardized image-capturing process was applied in each investigation. The instrument was equipped with an 830 nm continuous wave laser and a customized objective lens (P/N 04288, NA=0.9, Photon Gear), resulting in an imaging resolution corresponding to ~1.8 μm lateral and ~3 μm axial. Both Vivastacks and Vivablocks were acquired. Vivastacks were obtained by performing a series of 70-80 images in 3 μm steps to a depth of approximately 200 μm. Vivablocks up to 8x8 mm were composed of sequential RCM images at 500x500 μm each. In one LS patient, images were first acquired with RCM *in vivo* and then a skin biopsy was obtained from the area investigated with RCM. This biopsy was then complementary investigated using MPM technology *ex vivo* (Data S1).

Data analysis. The RCM images were acquired using the VivaScan software and exported as TIFF interface using mD4 (MAVIG GmbH). The acquired raw-Data images were subject to brightness and contrast enhancement using Photoshop (Adobe Systems Inc.) before assessment. All RCM images were evaluated by the same dermatologist (the first author). The complementary H&E-stained slides of the LS and plasma cell balanitis patients were evaluated by a pathologist specializing in dermatopathology (the second author). Then, the first and the second author together performed a more detailed comparison of the findings from RCM and the correspondence with histology, which accounts for the results presented.

Results

Patients and histopathology. In total, 30 males were included (17 with LS, 3 with plasma cell balanitis, 5 with nonspecific balanoposthitis, 1 with psoriasis and 4 healthy individuals). The clinical Data of the LS patients are presented in Table SI in the Data S1. All the LS patients had clinically active lesions. The cases with LS and plasma cell balanitis diagnosis were verified histopathologically. Furthermore, one patient with LS was histopathologically verified to have PeIN. In one patient with LS, an additional skin biopsy was taken after the examination with RCM, which was examined *ex vivo* with MPM. The MPM findings are presented in the Data S1.

All 17 biopsies from LS patient showed sclerosis histologically. In 12/17 samples the sclerosis was obvious and in 5/17 samples the sclerosis was mild.

RCM findings. The morphological features observed from the RCM investigation and the correlation with their histopathological counterparts are summarized in Table I.

A quantitative assessment of the characteristic RCM features observed is presented in Table II, comparing the Data acquired from the LS patients and the healthy group. The most significant features observed in LS were prominent fiber structures in the dermis, typical honeycomb pattern, irregular papillae and bright cells in the superficial dermis, while edged papillae, typical honeycomb pattern and irregular papillae were observed in all the health individuals.

The prominent fiber structures corresponding to hyaline sclerosis were observed in almost half (8/17) of the patients

Table I. Overview of the features observed by RCM and a comparison with their histopathological counterparts.

Histopathological features	RCM features
Normal epidermal architecture	Typical honeycomb pattern
Parakeratosis	Parakeratosis
Spongiosis/exocytosis	Exocytosis
Inflammatory cells in the dermis	Bright cells in the superficial dermis
Inflammatory cells inside the vessels in the dermis	Bright cells flowing inside the black lumen in the dermis
Irregular papillae	Irregular papillae
Normal papillary architecture	Edged papillae
Sclerosis in dermis	Prominent, fiber-like structures in dermis

RCM, reflectance confocal microscopy.

Table II. Overview and incidence of the features observed by RCM in LS and healthy penile skin.

RCM features	LS (incidence)	Healthy penile skin (incidence)
Typical honeycomb pattern	13/17	4/4
Parakeratosis	4/17	0/4
Spongiosis	9/17	0/4
Bright cells in basal layer	1/17	0/4
Bright cells in the superficial dermis	12/17	0/4
Bright cells flowing inside black lumen in the dermis	4/17	0/4
Irregular papillae	12/17	4/4
Dilated papillae	12/17	0/4
Edged papillae	2/17	4/4
Elongated papillae	5/17	0/4
Prominent fiber structures in dermis	8/17	0/4

LS, lichen sclerosus; RCM, reflectance confocal microscopy.

with LS (Table II). This feature is illustrated by Fig. 1. The cases in which RCM did not show sclerosis represented only mild sclerosis in histopathological examination. Interestingly, RCM could identify prominent fiber structures in one case of LS with mild sclerosis histopathologically. It should be noted that prominent fiber structures were observed in one of the plasma cell balanitis cases (Data S2, Table SII); however, this observation was done in a penile area where the patient had a scar and therefore deemed unrelated to the plasma cell balanitis.

The typical normal tissue architecture of the stratum spinosum observed as a honeycomb pattern in RCM (for illustration, Data S1, Fig. S1) was found in almost all patients including LS, in healthy individuals, nonspecific balanoposthitis, and plasma cell balanitis. Interestingly, in the LS patient who had histopathologically confirmed PeIN, the RCM investigation revealed an atypical honeycomb pattern and scattered, small, bright cells in the basal layer, probably corresponding to the cell dysplasia (Data S1, Figs. S1E and S2).

When investigating the dermo-epidermal junction, illustrated in Fig. 2, the healthy penile skin revealed edged papillae, representing rims of bright basal cells around the dermal papillae, corresponding to the normal papillary architecture, whereas in LS the edged papillae were absent or obscured.

Another feature observed using RCM was dermal inflammatory infiltrate observed as the presence of abundant bright cells in the dermis (Data S1, Fig. S3). This feature was observed in a majority of LS cases (12/17, Table II), while it was not found in healthy penile skin; however, it should be noted the feature was common feature in nonspecific balanoposthitis patients, why its diagnostic specificity for LS was low. In addition, RCM was also able to visualize bright cells flowing inside linear, canalicular structures in black lumen of the papillae in the dermis possibly representing the dilated vessels in the papillae (Data S1, Fig. S4). This was a common feature in patients with nonspecific balanoposthitis and in patients with plasma cell balanitis (Data S2, Table SII). Interestingly, this feature was uncommon in LS patients (Table II).

Among patients with plasma cell balanitis the most common feature was mildly refractive cells seen in the intercellular spaces (exocytosis) between keratinocytes, associated with

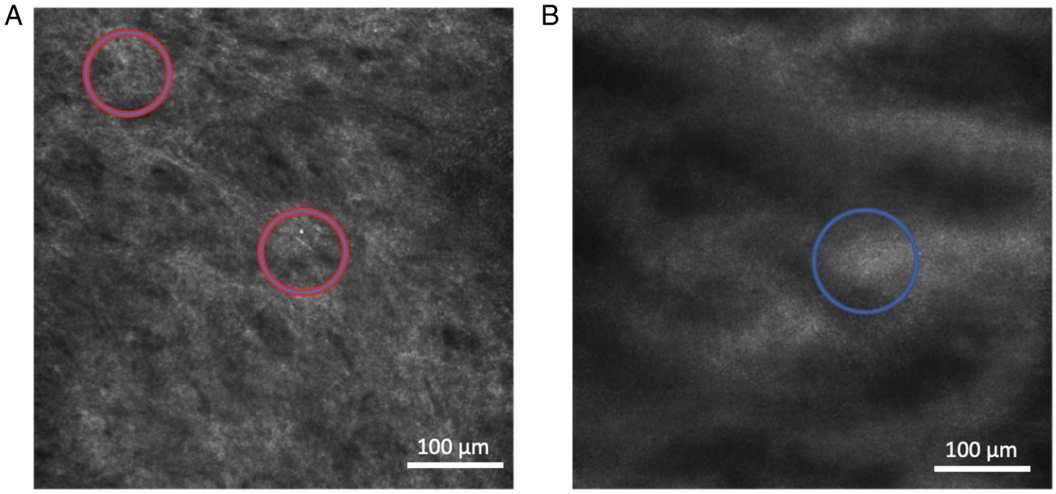

Figure 1. RCM Data acquired from (A) 1 patient with LS and (B) 1 healthy individual. As is shown in the figure, fiber structures were more prominent in the papillary dermis in (A) the patient with LS (red circles) than in (B) the healthy individual (blue circle). These prominent fiber-like structures represent sclerosis histopathologically, which can be visualized using RCM. Size of images, 0.5x0.5 mm. Scale bar, 100 μm. LS, lichen sclerosus; RCM, reflectance confocal microscopy.

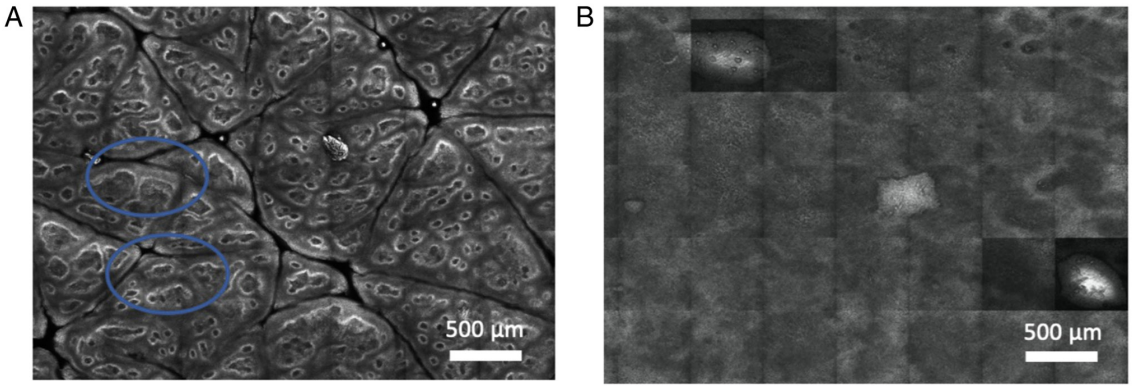

Figure 2. Reflectance confocal microscopy data acquired at the level of stratum basale from (A) 1 individual with healthy penile skin and (B) 1 patient with LS. (A) Edged papillae histologically representing normal papillary structures were observed in the healthy penile skin (blue circles), (B) whereas this feature was absent or obscured in LS where a flattening of the atrophic epidermis was observed. The edged papillae represent rims of bright basal cells around the dermal papillae. Both panels are a mosaic of 35 images, the size of every image is 0.5x0.5 mm. Scale bar, 500 μm. LS, lichen sclerosus.

spongiosis (Data S1, Fig. S5). Irregularity of the papillae in terms of their shape (Data S1, Fig. S6) was found in all groups, including the one with healthy individuals. An overview of the observed features in nonspecific balanoposthitis, plasma cell balanitis and psoriasis are shown in Table SII in the Data S2.

Complementary to RCM imaging, a tissue biopsy acquired from one of the patients was also investigated using MPM *ex vivo* (Fig. S7, Data S1). This is, to the best of our knowledge, the first time a case of LS has been investigated by MPM. The underlying principles of MPM is based on non-linear optical processes, makes it ideal to study collagen fibers. Consistent with histopathology, MPM revealed bright collagen fibers referring to sclerosis, but with greater contrast than the corresponding RCM image. Since sclerosis is a prominent feature signifying LS, this result implies that MPM would be an interesting complementary technique to visualize this feature in LS.

Questionnaire. In order to assess the patients' experience of the diagnostic procedure, the patients were asked to respond to a simple questionnaire. All but one patient answered that in order to receive a diagnosis, they preferred to be evaluated with RCM *in vivo* instead of undergoing a skin biopsy. RCM was experienced as painless, caused no discomfort and did not require local anesthesia. A majority, i.e., 12/17 LS patients, experienced the skin biopsy procedure as uncomfortable and unpleasant. The only reported disadvantage with RCM was that the procedure was time consuming (average time of investigation was ~30 min). This means that RCM investigation was overall well appreciated by the patients. According to the questionnaire, four LS patients had undergone complete circumcision and two LS patients had undergone partial circumcision before the examination with RCM.

Discussion

To date, RCM is a widely used technique in dermatology as a diagnostic tool for both tumors and inflammatory diseases (23,24). This study demonstrates the potential of RCM *in vivo* to visualize the characteristic histopathological features

of LS. The acquired RCM images were evaluated by a dermatologist and all the H&E-stained slides of the LS and plasma cell balanitis patients were evaluated by a pathologist specializing in dermatopathology. Complementary to earlier studies on the topic (20,21), this study includes an important comparison with images acquired from healthy penile skin, nonspecific balanoposthitis and plasma cell balanitis. In addition, the patient experience of the procedure was assessed by a questionnaire. Most of the LS patients described the skin biopsy procedure as unpleasant and preferred the non-invasive and painless RCM examination, supporting the clinical relevance of the study that there is a need of finding non-invasive diagnostic tools for the genital area and that RCM could fulfill this need.

Hyaline sclerosis is the key feature in the histopathological diagnosis of LS. In this investigation, this feature was observed as prominent, thick, fiber-like structures using RCM, and was found in almost half of the LS patients. Our results regarding sclerosis in LS patients was in line with other reports on genital and extragenital LS investigated with RCM (20,25). Similar, coarse, fiber-like structures in the dermis were noticed in a patient with plasma cell balanitis, however these represented a typical scar that the patient had in the area affected by plasma cell balanitis.

The most common feature identified in LS patients by RCM investigation was the typical honeycomb pattern along with the dilated and irregular papillae and bright cells in the dermis. These results agreed with those found by Lacarrubba *et al* (20). The comparison of the dermo-epidermal junction in the images obtained from LS and healthy penile skin, revealed edged papillae in the latter group. However, this feature was absent or obscured in the LS cases. The irregularity of the papillae and absence of edged papillae or non-rimmed papillae could indicate basal hydropic degeneration and loss of the melanogenesis of the basal cells, which are histological features found in LS. Reports support the fact that tumor necrosis factor-α and interleukin 17 act synergistically in inhibiting melanogenesis, thereby leading to the loss of melanin around the papillae causing the loss of the rims in inflammatory disorders such as psoriasis (26) and it could also explain the melanin loss in LS, which can be seen clinically as hypopigmentation. Moreover, the loss of edged papillae can be explained in certain cases due to the atrophic epidermis and the flattening of the junctional zone.

An inflammatory infiltrate in the dermis was identified in the majority of LS patients. Interestingly, bright cells flowing inside linear, canalicular structures in the black lumen of the papillae in the dermis was found more often in patients with nonspecific balanoposthitis than in LS. This feature may correspond to the dilated vessels found more commonly in balanitis than in LS. Nevertheless, neither the inflammatory infiltrate nor the dilated vessels seen with RCM are pathognomonic for nonspecific balanoposthitis or LS. Thus, these features cannot be used as diagnostic criteria for these disorders.

The investigation of one of the LS patients revealed an atypical honeycomb pattern in the epidermis and scattered round, nucleated, bright cells in the basal layer. These features are commonly found in squamous cell carcinoma *in situ* (20,27). The histopathological analysis of a skin biopsy obtained from the same area confirmed the diagnosis of PeIN. It is a common practice that LS patients are followed up regularly for signs of penile malignancy. To rule out cell dysplasia, skin biopsies are obtained from the already sensitive penile skin affected by LS. RCM is a diagnostic tool that can be used to evaluate nonmelanocytic skin tumors (28). More specifically, it has been used in order to differentiate between balanoposthitis and squamous cell carcinoma *in situ* (29), as well as to improve the diagnosis of oral carcinoma and its precursors (30). This study supports its use as a noninvasive monitoring tool for LS in risk of penile cancer and as a diagnostic tool for genital dysplasia.

In addition to RCM imaging, a tissue biopsy acquired from one of the patients was also investigated using multiphoton laser microscopy (MPM) *ex vivo* (Fig. S7, Data S1). This is, to the best of our knowledge, the first time a case of LS has been investigated by MPM. Complementary to RCM, MPM enables label-free imaging based on two-photon excitation and non-linear optical scattering (also known as second harmonic generation), making it ideal to study collagen fibers. Consistent with histopathology, MPM imaging revealed bright collagen fibers referring to sclerosis, but with greater contrast than the corresponding RCM image. *In vivo* MPM microscope is now commercially available, and it has been used to study skin tumors (31), although the technology has not yet been as clinically established as RCM. Based on the preliminary result in this study, an *in vivo* handheld MPM device could potentially be a more effective tool for visualizing sclerosis in the dermis and should be subject to further investigation in LS diagnostics.

The main limitation of this study is that sclerosis, which is the main characteristic of LS, was not identified in all LS patients examined by RCM. Several reasons account for this drawback. In three cases where RCM failed to detect the sclerosis, only mild sclerosis was seen histopathologically, thus making it difficult to be observed using RCM. However, the RCM was able to identify one case of mild sclerosis. Moreover, in some cases where the RCM investigation was performed several months or years after the skin biopsy was obtained, the patients received local treatment with potent steroid cream (5/17); the latter could have altered the typical histopathological features of LS and diminished the sclerosis making it more difficult to observe. In these cases, the histology is not directly comparable to the RCM findings. However, the participants were not allowed to use topical treatment 14 days prior to the RCM investigation. In addition, all the LS patients had clinical signs of active disease. Another factor that could have attributed to the absence of sclerosis features in RCM images obtained from LS patients could be the limitation of RCM to reach a skin depth of more than 150-200 μm. This limitation could be overcome by scanning the tissue with optical coherence tomography that has previously been used to study collagen fibers in LS and other conditions with excessive collagen deposition (32). In this study we did not have access to a hand-held RCM device, which could have simplified and hastened the imaging process. The image assessment was performed in an unblinded way that might have led to interpretation bias. Nevertheless, the images were evaluated in a standardized way at defined layers of the skin i.e., stratum corneum, stratum spinosum, stratum basale and dermis. The investigation was time consuming, and therefore it was not possible to examine all the affected area. Evaluating the produced images was also time consuming, in average three to four hours for every participant, making it difficult to implement RCM as a diagnostic tool in everyday clinical practice

today. In the future focus should be given in the application of machine learning-based image analysis on RCM and MPM Data to provide more quantitative and objective results (33). In addition, the images obtained using RCM present a horizontal view of the skin layers, thus making it difficult to directly correlate them with the histopathological images showing a vertical view of the skin. Furthermore, The RCM counterparts to skin atrophy and follicular hyperkeratosis were not evaluated in this study as they less commonly are present in genital LS. Another drawback of this study is the small number of healthy individuals included. Nevertheless, the RCM features observed on healthy penile skin were consistent, enabling us to clearly differentiate it from LS and nonspecific balanoposthitis.

In summary, our study showed that RCM could visualize the thick fiber-like structures corresponding to sclerosis in the dermis, confirming the previously reported findings on genital LS. In addition, we clearly showed the differences between healthy penile skin and LS by identifying the edged papillae on healthy skin and their absence or obscureness in LS patients. Importantly, RCM revealed a precursor of penile cancer in one LS patient.

In conclusion, RCM is a promising tool for diagnosing LS in a non-invasive manner. It can help discriminate LS from nonspecific balanoposthitis and plasma cell balanitis if sclerosis is present. Moreover, RCM could be a valuable tool for monitoring LS patients for dysplasia, reducing the number of follow-up biopsies and thereby eliminating potential complications.

Potentially, lasers scanning microscopy can become an important, and by patients well tolerated, non-invasive tool to detecting and mapping cell dysplasia, reducing the need for obtaining multiple biopsies form the penile area, thereby accelerating the treatment process by directly referring the patient to urologists for surgery.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DK interviewed all the participants in order to be included in the study, performed the microscopy imaging, obtained the skin biopsies, analyzed and interpreted the data, and wrote the manuscript. NN helped analyze and interpret the data, and contributed to the writing of the manuscript. KM helped to find patients with LS diagnosis to be included, interpreted the data and commented on the manuscript. AMWL interpreted the data and commented on the manuscript. PT contributed to analyzing and interpreting the data, as well as in compiling and writing the manuscript. DK and PT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Regional Ethics Review Board in Gothenburg (Dnr 415-17; Gothenburg, Sweden), and institutional rules for the clinical investigation of human subjects were followed. All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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