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Received: 22 December 2022 | Revised: 4 July 2023 | Accepted: 6 July 2023

DOI: 10.1111/exd.14888

RESEARCH ARTICLE

Experimental Dermatology WILEY

# Reflectance confocal microscopy as a non-invasive imaging tool in vulvar high-grade squamous intraepithelial lesions and lichen sclerosus: A descriptive morphological study in patients and healthy volunteers

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**Funding information**
Bontius Stichting, Grant/Award Number: 8222-32146

## Abstract

Incorrect and delayed diagnosis of vulvar high-grade squamous intraepithelial neoplasia (vHSIL) and lichen sclerosus (LS) increases malignant progression risks and negatively impacts prognosis and quality of life. There is a need to improve diagnosis and monitoring. Reflectance confocal microscopy is a non-invasive imaging tool that visualizes skin structures at cellular resolution. The objectives were to explore feasibility and patient acceptability of vulvar RCM imaging and to identify RCM characteristics that are discriminative for vulvar HSIL and LS. This was a prospective, cross-sectional, observational clinical trial in patients with vHSIL and LS compared to healthy volunteers. RCM images and vulvar tissue samples were obtained. Five (5) patients with vHSIL, 10 patients with LS and 10 healthy volunteers were enrolled. In total, 100 image series of vulvar skin were obtained, including lesional and nonlesional sites. The RCM technique was considered acceptable for application by patients and healthy controls. Healthy vulvar skin was characterized by a homogenous, normal honeycomb patterned epidermis and a clear epidermal-dermal junctions. Vulvar HSIL and LS displayed an atypical honeycomb pattern of the epidermis and lymphocytic influx with presence of melanophages. Distinct features specifically observed in LS included the presence of hyalinised vessels and sclerotic areas in the dermis. RCM is a non-invasive imaging technique that is feasible and clinically acceptable to apply on vulvar skin, both in patients with premalignant lesions and healthy controls. Recognition and validation of disease-specific characteristics could make reflectance confocal microscopy a clinical tool to non-invasively aid identification of vulvar premalignancies.

**KEYWORDS**
genital disease, lichen sclerosus, reflectance confocal microscopy, vulva

**Abbreviations:** dVIN:, differentiated vulvar intraepithelial neoplasia; HPV:, human papillomavirus; LS:, lichen sclerosus; RCM:, reflectance confocal microscopy; vHSIL:, vulvar high-grade squamous intraepithelial neoplasia; VSCC:, vulvar squamous cell carcinoma.

## 1 | INTRODUCTION

Incorrect or delayed diagnosis of vulvar high-grade squamous intraepithelial neoplasia (vHSIL) and lichen sclerosus (LS) has detrimental consequences as both can predispose to vulvar squamous cell carcinoma (VSCC) with estimated malignant progression risks of 5% and 10%, respectively.[1–3] vHSIL is caused by high-risk oncogenic human papillomavirus (HPV) infection, whereas the cause of LS is unknown.[4] LS can predispose to differentiated vulvar intraepithelial neoplasia (dVIN). This precursor lesion poses a considerable VSCC risk (33%–86%), indicating life-long LS surveillance and treatment.[3,5,6] Major issues in the management of vulvar premalignant disease include frequent misdiagnosis, delays in receiving correct diagnosis and recognition of disease margins. This delay is primarily caused by lack of awareness among patients and healthcare professionals, in addition to social stigma and taboo.[7–9] A five-year lag time is estimated between onset of pruritic symptoms and clinical LS identification.[10] Meanwhile, vHSIL and dVIN can be challenging to recognize even among specialized dermatologists and gynaecologists, necessitating pathological examination to reach a conclusive diagnosis.[11,12]

These diagnostic challenges illustrate the need for enhanced tools to improve timely recognition of vulvar diseases and prompt identification of malignant progression. One hypothesized tool is reflectance confocal microscopy (RCM). RCM is an in vivo imaging tool that uses a 830 nm laser to provide non-invasive, real-time and cellular level visualization of the epidermis and superficial collagen layers.[13] This technique has been applied for diagnosis of melanoma and basal cell carcinoma (BCC) and may reduce unnecessary biopsies of benign lesions.[14,15] Despite these applications, RCM imaging of the vulvar area has only been described in a few pilot studies to date.[16–21]

Therefore, the primary objective was to explore the technical feasibility and patient acceptability of RCM imaging on the vulva. The secondary aim was to describe morphological RCM characteristics that are discriminative for vHSIL and LS in comparison to nonlesional control sites as well as healthy females.

## 2 | MATERIALS AND METHODS

This RCM analysis was part of a single-centre observational clinical trial performed at the Center for Human Drug Research, Leiden, the Netherlands, from February 2021 to October 2021. The trial incorporated a multimodal range of techniques to identify and validate new clinical biomarkers for vHSIL and LS.[22,23] The study protocol was approved by the local ethics review board (Medisch-Ethische Toetsingscommissie Leiden Den Haag Delft) with reference number P.20.075. The trial was registered with the 'Nederlands Trial Register' (NL73964.058.20) and EudraCT (2020–002201-2). Subjects gave written informed consent prior to any study activities.

### 2.1 | Trial design and study population

In total, 25 women (Fitzpatrick skin type I-III) aged 25–72 were included. Ten healthy controls, five patients with vHSIL (≥1 sharply marginated histologically confirmed vHSIL lesion ≥15 mm) and ten patients with LS (clinical diagnosis) were enrolled. The washout for topically applied products on the vulvar area was ≥14 days. RCM images were obtained of visually lesional and (apparently) nonlesional sites of the vulva at every trial visit. Vulvar tissue was obtained using a 4 mm skin punch biopsy as histological reference in all participants. All patients and healthy volunteers completed a questionnaire assessing the patient acceptability of the RCM procedure compared to vulvar biopsies (range 0–100). The differences between procedures were analysed using a paired student's *t*-test for all subjects as one group in GraphPad version 9.3.1.

### 2.2 | RCM hardware and software

RCM images were obtained using the VivaScope confocal laser scan system per manufacturer instruction. Dermatoscopic images were obtained with the VivaCam® (VivaScope GmbH, Munich, Germany). The fixed VivaScope 1500 imaging module (Gen4) or the handheld VivaScope 3000 add-on imaging module were used for acquisition of RCM images (Figure S1). This resulted in cellular-resolution images which were analysed using the VivoScan software (Vivosight, Calibre I.D., Inc., Rochester, NY, USA).

### 2.3 | Scoring of RCM characteristics

The obtained RCM images were analysed by two raters (LP and BH) after completion of the clinical trial. All images were randomized and blinded (LP) before scoring by random assignment of a letter combination to the images sorted for subject type. As the raters had also obtained the RCM images, they cannot be considered fully blinded for patient type. Analysis was performed according to a predetermined set of characteristics in mutual agreement with MU and the raters (Table 1, Figure 1), based on knowledge from histology and RCM.[13,24–26] Training of the raters on recognition of RCM characteristics was performed by an expert in the field (MU) prior to full study analysis. Observations were summarized and shown descriptively.

### 2.4 | Morphologic analysis of vulvar reference tissue

Lesional and nonlesional vulvar tissue samples were obtained using a 4 mm punch biopsy acquired by trained physicians (LP, BH or MvP). Biopsies were formalin fixed and paraffin embedded (FFPE) and cut in 4 μm sections and stained for H&E. Pathological examination was performed by a dermatopathologist (JD).

**TABLE 1** Overview of selected morphological RCM characteristics based on literature.

| | Characteristic | Example | Scoring | Associated diseases | Description |
|---|---|---|---|---|---|
| Epidermis | Normal honeycomb | Figure 2A | Absent/Present | HV | A normal stratum granulosum and spinosum of the epidermis display a normal honeycomb pattern, made by the arrangement of the keratinocytes with demarcated outlines that make up a regular grid shaped like a honeycomb.[14] |
| | Atypical honeycomb pattern | Figure 2B | Absent/Present | vHSIL, LS, actinic keratosis, invasive squamous cell carcinoma and melanoma | Disarranged epithelial cells of different sizes, varying brightness of the lines without clear regular structure, as opposed to a normal honeycomb pattern.[14] |
| | Hyperkeratosis | Figure 2C | Absent/Present | LS, actinic keratosis, seborrheic keratosis, squamous cell carcinoma, warts and psoriasis | An increase of thickness stratum corneum, visible as reflectile amorphous material in RCM. Hyperkeratosis is commonly described in histological assessments of lichen sclerosus and vHSIL.[14,24,25] |
| DEJ/Dermis | Normal DEJ | Figure 2D | Absent/Present | HV | Basal cells are highly reflective and display a uniform size and shape. In the DEJ, the basal cells are arranged in a typical ringed appearance of dark round to oval shapes corresponding with the basal cell covering of the finger-like dermal papillae, a feature in RCM also called 'edged papillae'.[14] |
| | Disrupted DEJ | Figure 2E | Absent/Present | vHSIL and LS | Disappearance of the dermal papillae structure and degeneration of the basal layer has been described in lichen sclerosus.[24] |
| | Dermal sclerosis | Figure 2F | Absent/Present | Lichen sclerosus and lupus erythematosus | Thick and increased number of dermal fibres. Dermal sclerosus is a key feature of lichen sclerosus, also described in histological assessments.[14,25] |
| Immune infiltrate | Lymphocytic exocytosis | Figure 2G | Absent/Present | vHSIL and LS | Single or aggregates of round-to-polygonal, mildly refractive cells at the level of the stratum spinosum interspersed between keratinocytes.[14] Lymphoytes in the epithelium have been described in histological assessments of vulvar disease, indicating immune responses.[25,26] |
| | Lymphocyte infiltrate | Figure 2H | Absent/Mild/Moderate/Profound | vHSIL, LS and lupus erythematosus | Immune infiltrate in general, described in histological literature of both vHSIL (REF) and LS. This infiltrate can be observed in the dermis and can consist of different type of immune cells.[25,26] |
| | Melanophages | Figure 2I | Absent/Present | Chronic inflammatory diseases, for example, vHSIL and LS | Polygonal, bright structures larger than inflammatory cells and sometimes dendritic in the dermis. These cells are rich in melanin and are typically solitary distributed around papillary dermal capillaries.[14] Also described in histological assessments of lichen sclerosus.[25,26] |
| Vessels | Perivascular infiltrate | Figure 2J | Absent/Mild/Moderate/Profound | LS | Round-to-polygonal, mildly refractive cells around the dermal vessels. A perivascular distribution of atypical lymphocytes and inflammatory cells within dermal papillae may lead to a loss of the typical ringed appearance of dermal papillae at the DEJ.[14] Resulting from inflammatory process, often found in histological assessment of lichen sclerosus.[25,26] |
| | Ectatic vessels/capillaries in dermal papillae | Figure 2K | Absent/Present | LS | Canalicular vessels with bright cells characteristic of dilated, telangiectatic vessels.[14] Changes in vasculature due to sclerotic dermis and inflammatory process, also described in histology of lichen sclerosus.[25,26] |
| | Hyalinised or sclerotic vessels | Figure 2L | Absent/Mild/Moderate/Profound | LS | Stiff and notable blood vessels in the dermis, usually elongated with a pronounced vascular wall. Commonly described in histological assessment of lichen sclerosus.[25,26] |

## 3 | RESULTS

### 3.1 | Population characteristics

In total, 25 women, of which five patients with vHSIL, 10 patients with LS and 10 healthy volunteers were enrolled in the study. Baseline characteristics were comparable between patients and healthy controls, with mean ages of 46.6, 50.3 and 46.5 years, respectively (Table S1). Pre- and post-menopausal status was equally distributed within groups. All patients (vHSIL and LS) had previously undergone therapy for their vulvar disease. Histologically, all vHSIL and healthy tissue was confirmed corresponding to the clinical diagnosis. LS diagnosis was based on the clinical assessment, as stipulated by current protocols and guidelines. Three out of ten LS biopsies were morphologically confirmed as LS. The remaining being classified as normal skin with inflammatory reactive changes (e.g. acanthosis and lymphohistiocytic inflammation).

### 3.2 | Patient acceptability of RCM imaging

No adverse reactions were observed from the imaging oil or the adhesive from the plastic tissue cap on the vulvar area. Removal of the adhesive from the tissue cap was considered slightly uncomfortable for a short time without being painful and left no long-term irritation or pain. This was also the case for subjects with erosive lesions or image sites in sensitive areas like the labia minora or perianally. The non-invasive RCM procedure was considered significantly less burdensome compared to the invasive biopsy procedure ($p = 0.0259$) (Figure S2).

### 3.3 | RCM findings: morphological characteristics

In total, 100 RCM images were obtained at different study days and scored by two raters. Intentionally, a recording of each study day was analysed; however, some images were of poor quality and were therefore excluded. The final analysis image set included 29 of vulvar tissue of healthy control subjects ($N = 10$ HV), 12 recordings of lesional vHSIL ($N = 5$ patients), two of nonlesional skin of vHSIL patients, 42 of lesional LS vulvar skin ($N = 10$ patients) and 15 of nonlesional skin of LS patients ($N = 10$) mostly of the groin region as LS often involved the whole vulvar area. A representative example of all characteristics is shown in Figure 1. For each population (healthy control, vHSIL and LS) a representative case with disease-distinguishing characteristics is shown to allow for side-by-side comparison of corresponding histological, dermatoscopic and RCM images (Figures 2–4 and Figure S3).

Features characterizing healthy skin were an intact dermal-epidermal junction (100%) and a normal, honeycomb patterned epidermis, generally in the absence of inflammatory cells, unusual vessel structure and epidermal or dermal changes. Some of the scored characteristics were occasionally observed, such mild lymphocytic infiltrate in the dermis (Figure 2).

Common characteristics identified in vHSIL were an atypical honeycomb pattern (75%) and presence of melanophages (62.5%). Lymphocyte infiltration was mildly to profoundly present in 71%, mostly in the dermis but also with epidermal lymphocytic exocytosis in 37.5% of cases. No dermal sclerosis was identified. Vascular changes were minimal (Figure 2). Only two recordings were obtained on nonlesional vulvar skin of vHSIL, of which one was of insufficient quality to assess, whilst the other was classified as healthy vulvar skin without notable characteristics or morphological changes.

Ectactic (63%) or hyalinised sclerotic vessels (68%) and dermal sclerosis (71%) were among often observed characteristics in LS. In addition, mild to profound lymphocyte infiltrate (79%) was found in the dermis. An atypical honeycomb pattern was observed in 56% of the LS epidermis analysed. In 88% of cases, the dermal-epidermal junction was considered intact (Figure 2). Nonlesional LS tissue was characterized by the absence of honeycomb atypia or immune cell influx. In a minority of cases (7%), dermal sclerosis and hyalinised vessels were observed (Figure S4).

The overall observation by the operators was that the presence of RCM features corresponding with lesional vulvar skin could generally differentiate diseased from healthy vulvar skin. However, these findings currently could not reliably discriminate vHSIL from LS (Figure 2). Immune cell influx and atypical honeycomb patterns of the epidermis were observed at comparable rates in vHSIL and LS. Dermal sclerosis and sclerotic vessels were distinctively identified in LS.

## 4 | DISCUSSION

This is one of the first studies to systematically assess and extensively describe the application of RCM imaging on the vulvar area. We have shown that RCM is technically feasible on the vulva of patients with premalignant disease with minimal patient discomfort. In addition, we were able to identify and compare RCM features of healthy vulvar skin to vHSIL and LS. RCM features observed in vHSIL included an atypical honeycomb pattern of the epidermis and strong dermal lymphocytic influx including melanophages. Lymphocytic exocytosis into the epidermis may also be present. These features were also observed in LS, with the distinct presence of sclerotic, collagenous areas and pronounced, hyalinised vessels in the dermis of LS vulvar skin. In line with previous findings, our control group included healthy vulvar skin that showed a normal honeycomb pattern of the epidermis and a distinct dermal-epidermal junction (DEJ).[17,20]

This study adds to the sparse available data of the application of RCM in vHSIL, LS and healthy vulvar skin. Fouques et al. were the first and only to report on RCM features of 10 patients with lesions suspicious for vHSIL.[20] They described features of vHSIL including an atypical honeycomb pattern, parakeratosis and keratinocyte atypia. This concurs with our findings, although lymphocytic influx, a common (71%) feature in our analysis, was not described. In addition, we did not include parakeratosis or keratinocyte atypia in our analysis. In three LS lesions, Fouques et al. reported a normal

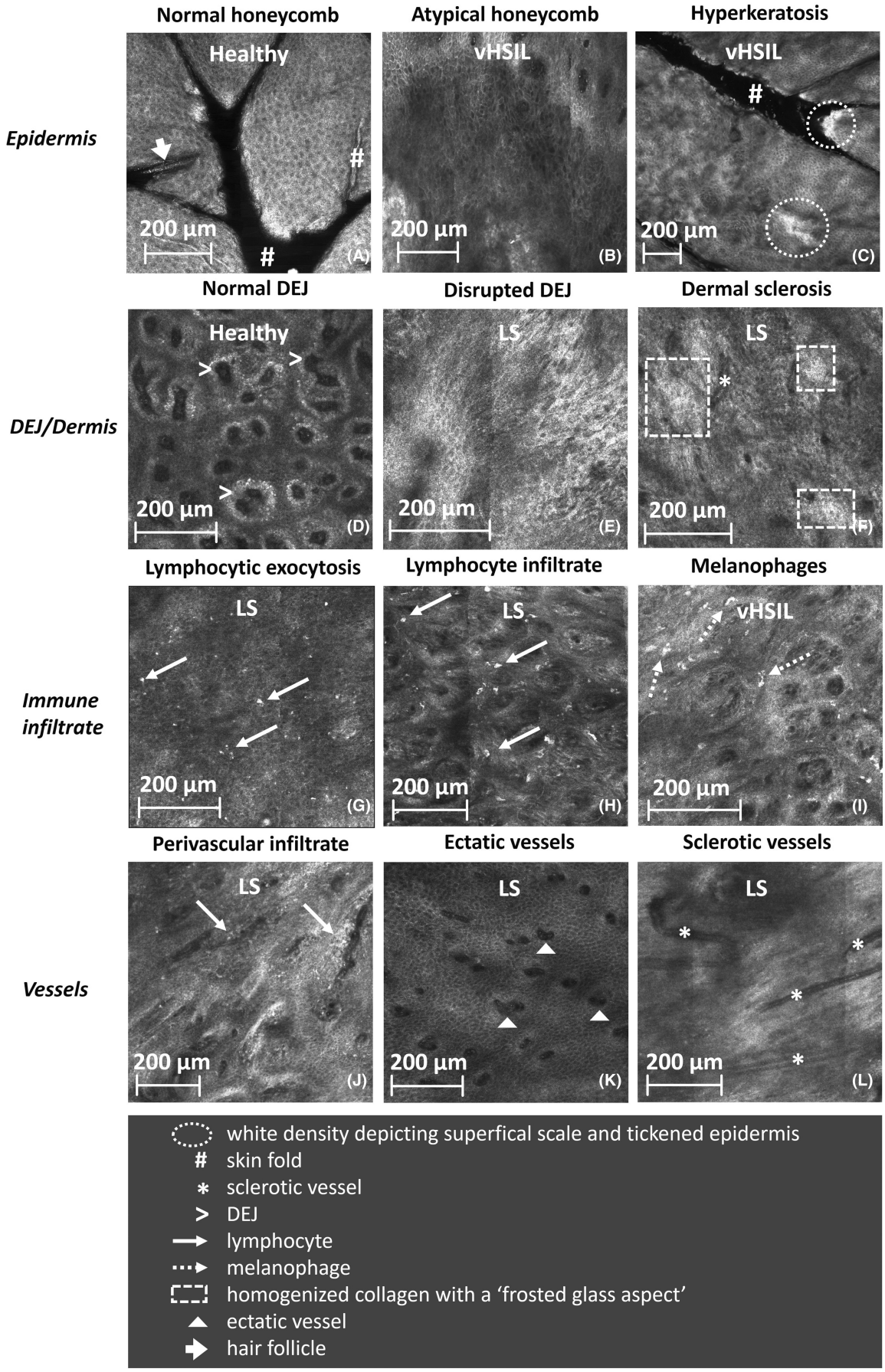

**FIGURE 1** Representative images showing examples of scored characteristics. (A) Normal honeycomb pattern of the epidermis of labia majora (Healthy)—depth 60 μm; (B) Atypical honeycomb pattern of the epidermis of the perineum (vHSIL)—depth 85 μm; (C) Hyperkeratosis in the epidermis of the perineum (vHSIL)—depth 27 μm; (D) Normal pattern of the dermal-epidermal junction (DEJ) of the labia majora (Healthy)—depth 73 μm; (E) Absent or disturbed dermal papillae located in the labia majora (note the lack of papillary pattern and direct transition from epidermal cells (bottom left) to sclerotic dermis) (LS)—depth 195 μm; (F) Dermal sclerosis (or homogenized collagen) in the dermis of the labia minora (LS)—depth 83 μm; (G) Lymphocytic exocytosis in the epidermis of the perineum (LS)—depth 61 μm; (H) Profound lymphocytic infiltrate in the dermis of the labia minora (LS)—depth 76 μm; (I) Presence of melanophagesin the epidermis of the interlabial fold, paraclitoral (vHSIL)—depth 91 μm. (J) Perivascular infiltrate in the dermis of the labia majora (LS)—depth 178 μm; (K) Ectactic vessels in the dermal papillae of the interlabial fold, paraclitoral (LS)—depth 78 μm; (L) Sclerotic vessels in the dermis of the inner side of the labia minora (LS)—depth 106 μm. Scale bars in all images represent 200 μm.

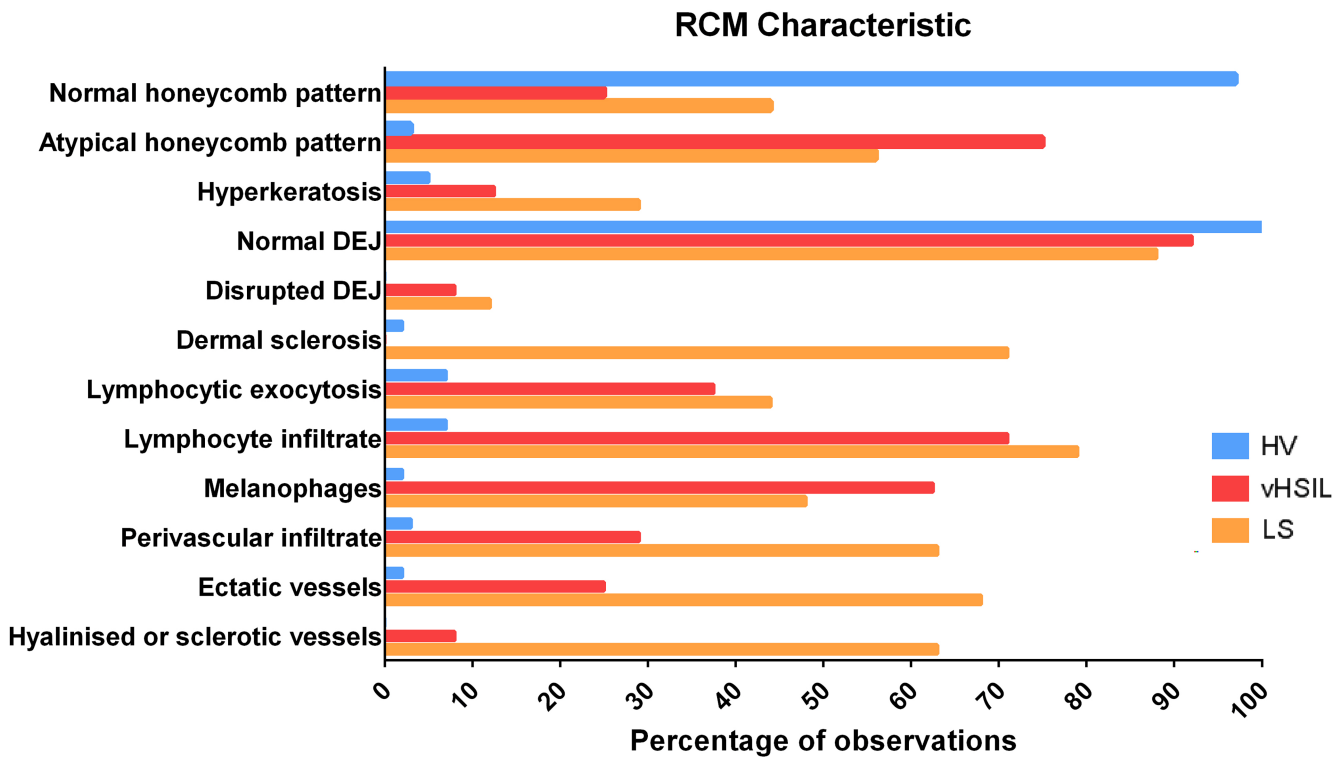


**FIGURE 2** Percentage observed characteristics in RCM images, in HV, vHSIL and LS. The average percentage of observations of both two raters were shown. HV, healthy volunteer, $n = 58$ observations; vHSIL, vulvar HSIL, $n = 24$ observations; LS, Lichen sclerosus, $n = 84$ observations. DEJ, dermal epidermal junction.

honeycomb epidermal pattern and a 'frosted glass aspect' (dermal sclerosis) of the dermis. We confirmed these findings in a more substantial population, but also identify honeycomb pattern disruption in 56% of cases. Dermal sclerosis was considered one of the prominent features of LS in our findings. Melanocyte influx in 3/6 cases of hyperpigmented LS were reported by Theillac et al., concurring with our finding of 48% melanocyte presence in LS.[27] Vascular changes, which were identified in 63%–68% of our LS cases, have not previously been described. The remaining literature on the application of RCM on the vulva is limited by case studies which primarily focus on pigmented lesions.[16–19,28,29]

The main strength of this study is the inclusion of healthy controls as reference for scoring the characteristics of vHSIL and LS skin. Within-patient control potentially confounds 'healthy' findings as the tissue can be compromised by scarring, immune cell infiltration or treatment effects. As previous studies lacked a detailed description of the application of RCM on (diseased) vulvar tissue, we here showed our practical considerations and a setup to facilitate follow-up studies by other research groups. Also, although the handheld RCM tool is useful for difficult to reach vulvar areas, this study shows that larger scanning areas using the 1500 scanning head is feasible and renders high-quality images with direct dermatoscopic reference. Patient acceptability assessment scoring as incorporated in this study is essential to encourage further development and clinical integration of the technique.

Limitations of this study include the modest sample size which means that our assessments require replication in a larger and more diverse patient population (including VSCC and dVIN) to appraise validity of our findings, repeatability and clinical application. We were unable to recruit patients with dVIN or VSCC, which would have enabled comparison of RCM features between premalignant vulvar tissue and invasive VSCC. Another limitation concerns that the data were assessed by only two raters. In total, 100 images were obtained from 25 subjects, thus no corrections for repeated

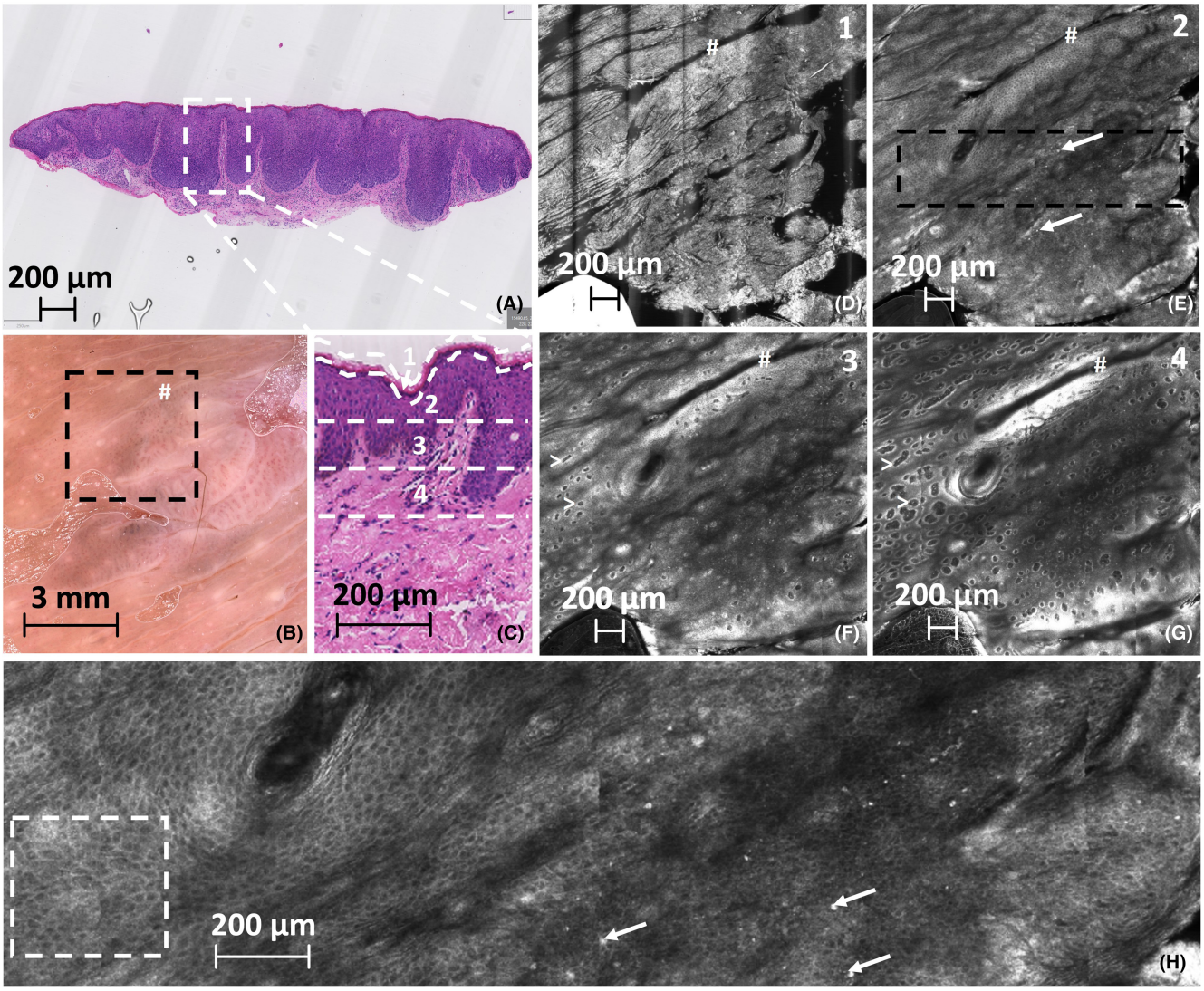


FIGURE 3 Images of representative lesional skin of vHSIL in the perianal area. (A) HE staining of a 4 mm punch biopsy of lesional warty-type vHSIL on the perineum. Scale bar represents 200 µm. (B) Macroscopic dermatoscopic image of a vSHIL lesion in the peri-anal area of another patient (more representative corresponding RCM images). The black insert represents the location of the RCM close-ups represented in image C–G. Scale bar represents 3 mm. (C) Insert of a HE staining as annotated in A. Dashed areas 1, 2, 3 and 4 represent skin layers stratum corneum, stratum granusolum, stratum spinosum and epidermal-dermal junction (EDJ), respectively, matching to the layers shown in RCM images D–G. Scale bar represents 200 µm. (D) RCM image of the stratum corneum with skin folds (# signs) (depth 0 µm). (E) RCM image of the epidermis showing an atypical honeycomb pattern and lymphocyte influx with melanophages (white arrows) (depth 41 µm). (F) RCM image showing the epidermal-dermal junction (> sign) (depth 81 µm). (G) RCM image showing the dermis and the dermal papillae with the epidermal-dermal junction (> sign) (depth 121 µm). (H) RCM image showing a magnification of the indicated area (black dashed box) in RCM image E displaying an atypical honeycomb pattern (white dashed box) and lymphocytic influx (bright white cells) including melanophages (white arrows). The scale bars in the RCM images (D–H) represent 200 µm.

within-subject observations were performed in this descriptive analysis. Additionally, this study stratified described RCM features by disease, and not by anatomical location, although the vulva is a diverse anatomical structure from the hirsute labia majora to the mucosal vaginal vestibulum.

LS is diagnosed based on clinical features. This could raise valid concerns for the clinical applicability of RCM for this benign disease. In our opinion, the potential gain of the use of RCM lies in earlier and improved non-invasive recognition of lesions suspicious for dVIN or VSCC during the life-long follow-up of LS patients. In addition, RCM has been shown to enable safe reduction in biopsy frequency in basal cell carcinoma.[30] On the vulva, this potential benefit could be even more vital in sparing essential vulvar structures such as the clitoris and urethra. This could also apply for the recognition and follow-up of vHSIL, dVIN and VSCC, which are diagnoses that currently require histological confirmation. In addition, RCM might help to identify the most aberrant part of the vulvar lesion for choosing the most suspicious biopsy location. Before incorporation into the dermatological or gynaecological practice, a more expansive, prospective and long-term study of

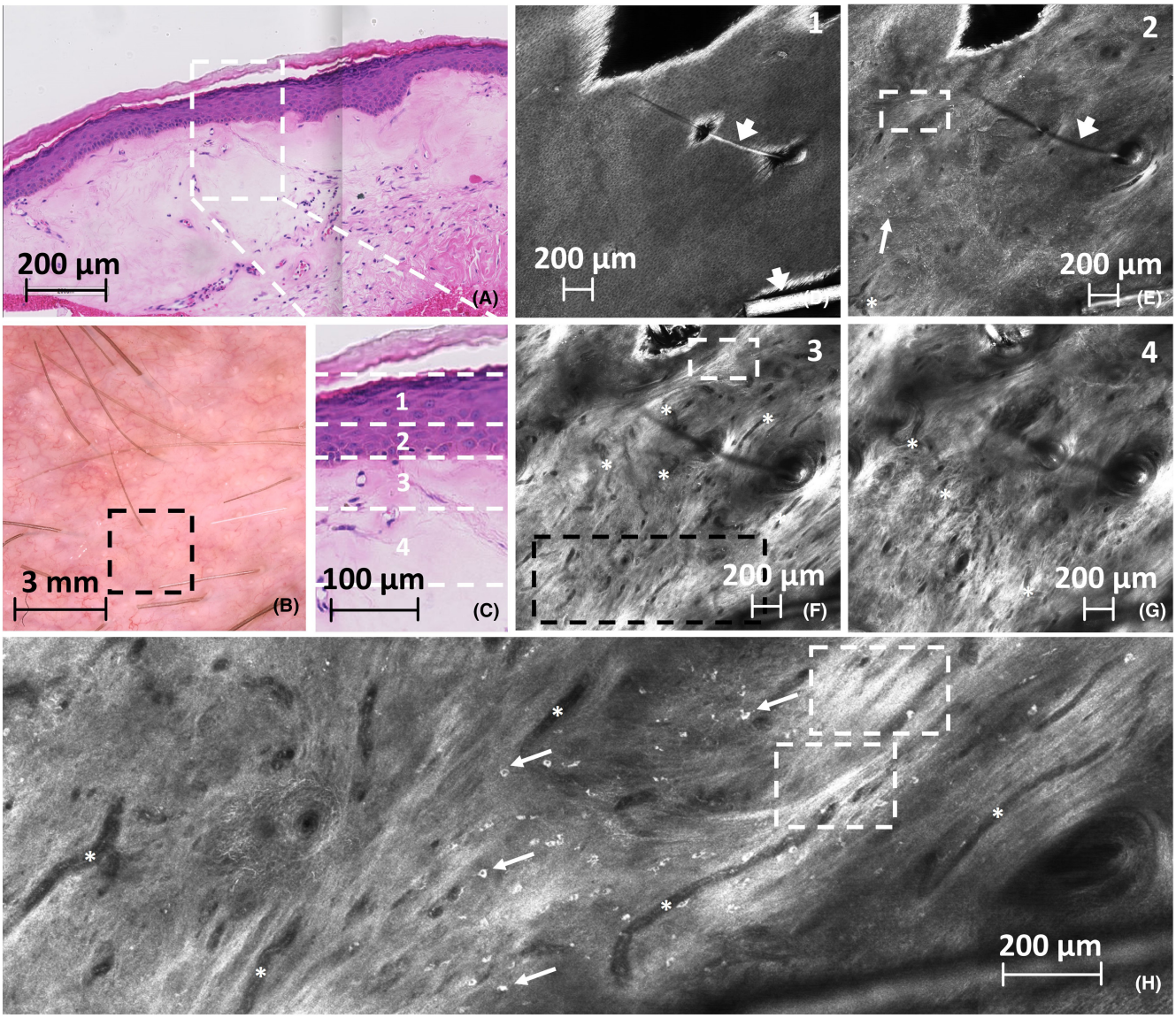


**FIGURE 4** Images of representative vulvar skin of a LS patient—labia majora inner side. (A) HE staining of 4 mm punch biopsy of lesional LS skin on the inner side of the labia majora. Scale bar represents 200 µm. (B) Macroscopic dermatoscopic image of correspondig lesional biopsy (LB) location with black insert of the close-up of the RCM images in D–G. Scale bar 3 mm. (C) Insert of HE staining represented in A. Dashed areas 1, 2, 3 and 4 represent skin layers stratum corneum, stratum granusolum, stratum spinosum and epidermal-dermal junction (EDJ) respectively, correlating to the layers shown 1–4 in the RCM images D–G. Scale bar represents 100 µm. (D) RCM image at the stratum corneum with a visible hair (arrowhead) (depth 29 µm). (E) RCM image of the transition from the epidermis to the dermis showing an atypical honeycomb pattern in the epidermal structure, loss of dermal papillae, melanophage influx (white arrows), sclerotic vessels (asterixes) and sclerotic dermal areas (white dashed box) (depth 78 µm). (F) RCM image showing the dermis with pronounced sclerosis (white dashed box) and hyalinised, sclerotic vessels (asterixes) (depth 126 µm). (G) RCM image showing the deep dermis with distinct sclerosis (white dashed box) and hyalinised vessels (asterixes) (depth 175 µm). (H) RCM image showing a magnification of the indicated area (black dashed box) in RCM image F displaying moderate to profound lymphocytic (bright white cells) and melanocytic infiltration (white arrows), sclerotic areas (white dashed box) and stiff, hyalinised vessels (asterixes). The scale bars in the RCM images (D-H) represent 200 µm.

vulvar features of RCM should be conducted, including all suspicious lesions in the outpatient clinic, including vHSIL, LS, dVIN and VSSC. Hence, the RCM features in lesions ranging from healthy vulvar skin to invasive cancer could be collected and validated to create a scoring and reference system for clinicians. Interstitial lymphocyte infiltration, follicular plugging and basal membrane thickening are typical histological features of LS that were not included in the preset of characteristics scored in this analysis, but could be considered for follow-up studies.[26] Similarly, keratinocyte atypia was described in a case study in penile intraepithelial neoplasia, which could be used for future RCM studies to vHSIL.[31] To facilitate image interpretation, we suggest standardizing the image acquisition protocol, stipulating the depth interval between images and the areas of special interest.

In conclusion, RCM imaging on the delicate vulvar area is feasible and well tolerated by patients, also in premalignant vulvar diseases. Presence of morphological RCM characteristics observed in the RCM images of this study have the potential to distinguish vHSIL and lichen sclerosus from healthy vulvar skin. However, a discriminative set of RCM features that could facilitate vulvar disease diagnosis was could not consistently be identified. This would require expansion of patient groups and inclusion of additional vulvar disease entities. This technique could lead to improved diagnostics of premalignant lesions and potentially guide or reduce biopsy frequency, though clinical validation in larger patient groups with long-term follow-up is crucial before implementation. Future studies should also focus on elucidation of RCM characteristics of dVIN and VSCC to improve recognition of the complete dysplastic pathway from healthy vulvar skin to invasive malignant disease.

## AUTHOR CONTRIBUTION

BH, LP, RR, TN and MvP designed the research study. JD, JP and MvP performed clinical and pathological assessments and aided patient recruitment. BH and LP performed the clinical research; BH, LP and MU analysed the RCM images. MU, RR, JP, TN and MvP supervised the study and aided interpretation of data; BH and LP wrote the manuscript. All authors have read and approved the final manuscript.

## ACKNOWLEDGEMENTS

The authors would like to thank all patients and healthy volunteers for their participation. The patients in this manuscript have given written informed consent to publication of their case details. The authors thank the 'Stichting Lichen Sclerosus' for their aid in patient recruitment. The authors thank the donors of the Bontiusstichting (project 8222-32146) of the Leiden University Medical Centre for their financial contribution to clinical research.

## FUNDING INFORMATION

This study was funded by the CHDR R&D fund and partially supported by the Bontiusstichting (project 8222-32146) of the Leiden University Medical Centre.

## CONFLICT OF INTEREST STATEMENT

The authors BH, LP, MU, RR, JD, TN and MP have no competing interests to declare. JP has received grants for projects outside the scope of this article from Philips, KWF, Catharina Onderzoeksfonds and Ruby and Rose.

## DATA AVAILABILITY STATEMENT

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

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Figure S1** (A) In vivo VivaScope 1500/3000 system. Coloured annotations indicate different components of the system: Vivascope 1500 scan head (green), VivaCam (red), Vivascope 3000 handheld add on (orange), overview picture of the assessed subject (blue), real-time RCM image (pink) of a depicted area of the macroscopic image (purple) of a small area of the overview picture. (B) Imaging frame of the software showing the macroscopic overview picture of the assessed healthy subject (blue), real-time RCM image (pink) of a depicted area of the macroscopic image (purple) of a small area of the overview picture. (C) Setup during a study day, including a gynaecologic chair for imaging of the vulva with the Vivascope in an acceptable setting for both the operator and the subject. With the Vivascope 1500 scan head (green) in study position. Placement of the RCM tissue cap on (D) the labia minora of an LS patient with the adhesive on the skin. (E) the labia majora of a healthy volunteer with the adhesive partially placed on the skin and held in place by the operator.

**Figure S2** Mean (SD) patient rating on a visual numeric rating scale (NRS) of 0–100 answering the question 'In your experience, how burdensome did you find the assessment?', shown per patient group. HV, healthy volunteer, vHSIL, vulvar HSIL, LS, Lichen sclerosus. Assessments shown are RCM = reflectance confocal microscopy and biopsy = vulvar biopsy. Asterisk (*) signifies a p-value of 0.0259.

**Figure S3** Images of representative healthy vulvar skin on the labia majora. (A) HE staining of a split 4 mm punch biopsy of vulvar labia majora. Scale bar represents 200 μm. (B) Macroscopic dermatoscopic image of corresponding nonlesional biopsy (NLB) location with black insert of the close-up of the RCM images in D–G. Scale bar represents 3 mm. (C) Insert of HE staining represented in A. Dashed areas 1, 2, 3 and 4 represent skin layers stratum corneum, stratum granusolum, stratum spinosum and epidermal-dermal junction (EDJ), respectively, correlating to the layers shown 1–4 in the RCM images D–G. Scale bar represents 100 μm (D) RCM image at the stratum corneum with skin folds (# sign) (depth 0 μm). (E) RCM image of the epidermis showing a normal honeycomb pattern (depth 30 μm). (F) RCM image showing the epidermal-dermal junction (> sign) (depth 61 μm) (G) RCM image showing the dermis and the dermal papillae with the epidermal-dermal junction (> sign) (depth 91 μm). The scale bars in the RCM images (D–G) represent 200 μm.

**Figure S4** Percentage observed characteristics in RCM images, in nonlesional LS. The average percentage of observations of both two raters were shown. LS, Lichen sclerosus, n = 29 observations. DEJ, dermal-epidermal junction.

**Table S1** Baseline characteristics.

**How to cite this article:** Huisman BW, Pagan L, Ulrich M, et al. Reflectance confocal microscopy as a non-invasive imaging tool in vulvar high-grade squamous intraepithelial lesions and lichen sclerosus: A descriptive morphological study in patients and healthy volunteers. *Exp Dermatol*. 2023;32:1734-1743. doi:10.1111/exd.14888