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Taking a closer look: non-invasive tools for in-depth characterisation of vulvar diseases

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Citation

Pagan, L. (2023, December 12). *Taking a closer look: non-invasive tools for in-depth characterisation of vulvar diseases*. Retrieved from <https://hdl.handle.net/1887/3666289>

Version: Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

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

Published in Experimental Dermatology, 2023. Doi: 10.1111/exd.14888

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Abstract

BACKGROUND Incorrect and delayed diagnosis of vulvar high-grade squamous intraepithelial neoplasia (vHSIL) and lichen sclerosis (LS) increases malignant progression risks and negatively impacts prognosis and quality of life. There is a need for novel techniques to improve diagnosis and monitoring. Reflectance confocal microscopy is a non-invasive imaging tool that can visualize skin structures at cellular resolution. However, reflectance confocal microscopy has not extensively been described on vulvar HSIL or LS.

OBJECTIVES The primary objective was to explore feasibility and patient acceptability of RCM imaging on premalignant vulvar skin. The secondary aim was to identify RCM-characteristics that are discriminative for vHSIL and LS.

METHODS This was a prospective, cross-sectional, observational clinical trial in patients with vHSIL and LS compared to healthy volunteers. RCM recordings and vulvar tissue samples were obtained.

RESULTS Five (5) patients with vHSIL, 10 patients with LS and 10 healthy volunteers were enrolled. In total, 100 recordings of vulvar skin were obtained, including lesional and non-lesional sites. The reflectance confocal microscopy technique was considered acceptable for application by patients and healthy controls. Healthy skin was characterized by a homogenous, normal honeycomb patterned epidermis and a clear epidermal-dermal junctions. Vulvar HSIL and LS lesions often 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.

CONCLUSIONS Reflectance confocal microscopy 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. However, studies to validate disease-specific characteristics in a wider range of vulvar diseases including vulvar squamous cell carcinoma are indicated.

Introduction

Incorrect or delayed diagnosis of vulvar high-grade squamous intraepithelial neoplasia (vHSIL) and lichen sclerosis (LS) has detrimental consequences as both diseases can predispose to vulvar squamous cell carcinoma (VSCC). Malignant progression risks are estimated to be up to 5% and 10%.¹⁻³

vHSIL is caused by high-risk oncogenic human papillomavirus (HPV) infection and symptoms include genital pruritus or pain. Peak prevalence is in women aged 20-35 years.^{3,4}

Genital LS has a peak incidence in woman aged 45-60 years, but also occurs in prepubertal children and adult males.⁵

LS is characterized by vulvar pruritus. Clinically, the vulvar skin becomes thinner, less flexible, and hypopigmented or erosive areas appear in a classic 'figure of eight' shape. LS is a chronic disease with mutilating effects due to disappearance of the labia minora and clitoris and vaginal narrowing. Current standard-of-care treatment for LS is topical application of ultra-potent corticosteroids, which can reduce VSCC incidence if applied with high compliance.⁶

The malignant pathway from LS to VSCC usually progresses via a precursor lesion known as differentiated vulvar intraepithelial neoplasia (dVIN). The malignant transformation risk is very high (33-86%) with cancer progression usually occurring within 2 years after dVIN diagnosis.^{3,7}

For these reasons, life-long surveillance and therapy is indicated for LS patients.

Major issues in the management of vulvar premalignant disease include frequent misdiagnosis, delays in receiving the correct diagnosis and the recognition of disease margins for biopsy or therapy. This delay is mainly caused by a lack of awareness among patients and healthcare professionals, in addition to the social stigma and taboo that patients experience causing a delay in time from symptom occurrence to presenting to a physician.^{8,9,10} Even among specialised dermatologists and gynaecologists, vulvar premalignancies such as vulvar HSIL and dVIN are sometimes challenging to recognise. A pathological examination of invasively obtained biopsy material is needed for a conclusive diagnosis of vulvar HSIL and dVIN.^{11,12} For LS, the mean time between onset of pruritic symptoms and identification is reported to be approximately 5 years. It is assumed that the onset of disease occurs even earlier.¹³ These diagnostic challenges illustrate the need for enhanced tools

to improve timely recognition of vulvar diseases and prompt identification of malignant progression. One of our hypothesised tools is the application of non-invasive, real-time techniques such as reflectance confocal microscopy (RCM). RCM is an *in vivo* confocal imaging tool that uses a low powered laser (830nm) to provide non-invasive and real-time visualization of the epidermis and superficial collagen layers at a cellular level up to a depth of 150 μm .¹⁴ This results in optical transversal sectioning of unstained epithelium and stroma. This technique has been applied for early and accurate diagnosis of skin tumors including melanoma or basal cell carcinoma (BCC) and reportedly may reduce unnecessary biopsies of benign lesions.^{15,16} Despite these technological and clinical advancements in the improvement of diagnostic accuracy, RCM imaging of vulvar area has only been described in few pilot studies.¹⁷⁻²⁰

These studies lacked thorough descriptions of methodology and specifics of clinical application of RCM on the delicate vulvar area. Only one small study has previously described vHSIL using RCM imaging and none to date have described vulvar LS.²¹ The primary objective of this clinical trial 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 non-lesional control sites as well as healthy females.

Methods

This RCM analysis was part of a single-centre observational clinical trial performed at the Centre for Human Drug Research in Leiden, The Netherlands, from February 2021 to October 2021. The trial incorporated a multi-modal 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 'Netherlands Trial Register' (NL73964.058.20) and EudraCT (2020-002201-2). Subjects gave written informed consent prior to any study activities.

TRIAL DESIGN AND STUDY POPULATION

In total, 25 women (Fitzpatrick skin type I-III), aged 25-72 with a body mass index (BMI) $<30 \text{ kg/m}^2$ were included. Ten healthy controls, five patients with vHSIL (≥ 1 sharply marginated histologically confirmed vHSIL lesion $\geq$

15mm) and ten patients with LS (clinical diagnosis) were enrolled. The wash-out for topically applied products on the vulvar area was ≥ 14 days. RCM images were obtained of visually lesional and (apparently) non-lesional sites of the vulva at every trial visit. Vulvar tissue was obtained using a 4mm skin punch biopsy as histological reference. 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.

PREPARATION AND OBTAINING OF RCM IMAGES

RCM images were obtained using the VivaScope confocal laser scan system, with the VivaCam® (VivaScope GmbH, Munich, Germany) for dermatoscopic images and the fixed VivaScope 1500 imaging module (Gen4) or the hand-held VivaScope 3000 add-on imaging module for microscopical images (*Supplementary Figure 1A+B*). The patients were seated in the gynaecological chair to obtain the RCM images (*Supplementary Figure 1C*). Generally, one operator is required to obtain an RCM image, but in this study most images were obtained by two operators (LP and BH) assisting each other.

A small drop of imaging oil (Crystal Plus Food Grade Mineral Oil FG-40z, Vivascope) was placed between the skin and the plastic imaging frame. Areas of interest included the labia majora, labia minora, interlabial fold, perineum and peri-anal sites. Hairs were occasionally clipped to enhance image quality. Generally, the adhesive of the plastic tissue cap was applied to the vulvar skin to keep the cap in place during the imaging procedure (*Supplementary Figure 2D+E*). In case of disrupted, sensitive vulvar skin, the protective cover paper on the adhesive area was partly removed and/or the tissue cap was held in place by one operator while the other operator executed the imaging steps. A dermatoscopic image was obtained for reference and orientation using the VivaCam. Subsequently, water soluble and hypoallergenic ultrasound gel (Aquasonic, Parker) was placed within the tissue cap and the Vivascope 1500 scanner was placed in the cap. The imaging scanner moved within the tissue cap as they were navigated across the vulvar skin via the computer-driven instructions of the operator based on the macroscopic image. This resulted in cellular-resolution images of the epithelium of the skin and supporting stroma using the VivoScan software (Vivosight, Caliber I.D., Inc., Rochester, NY, USA). Images were obtained with functions 1) VivoBlock: single image of 0.5

x 0.5mm at a pre-selected depth; 2) VivoStacks: vertical series of images at the same horizontal position and 3) VivoCube: series/stack of RCM images/blocks digitally rendering a mosaic up to 3x3mm. In case of difficult to reach vulvar areas, the hand-held VivaScope 3000 system was used without dermatoscopy alignment to obtain RCM footage. Complete image acquisition took 5-15 minutes, depending on the size and number of the images.

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. Analysis was performed per location according to a pre-determined set of characteristics in mutual agreement with MU and the raters (*Table 1, Figure 1*), based on knowledge from histology and RCM.^{14,24-26}

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.

MORPHOLOGIC ANALYSIS OF VULVAR REFERENCE TISSUE

Lesional and non-lesional 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).

Results

POPULATION CHARACTERISTICS

In total, 25 women, of which 5 patients with vHSIL and 10 patients with LS and 10 HV 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 (*Supplementary Table 1*). Pre- and postmenopausal 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, lymphohistiocytic inflammation).

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 peri-anally. The non-invasive RCM procedure was considered significantly less burdensome compared to the invasive biopsy procedure ($p=0.0259$) (*Supplementary Figure 2*).

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), 2 of non-lesional skin of vHSIL patients, 42 of lesional LS vulvar skin (N=10 patients) and 15 of non-lesional 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, LS) a representative case with disease-distinguishing characteristics is shown to allow for side-by-side comparison of corresponding histological, dermatoscopic and RCM images (Figs. 3-5).

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 non-lesional 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*). Non-lesional 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 (*Supp Figure 3*).

Discussion

This is the first study 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).^{18,21}

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.²¹ 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 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.^{17-19,28-30}

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 hand-held 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 pre-malignant vulvar tissue and invasive vSCC. Another limitation concerns that the data was assessed by only two raters. In total, 100 images were obtained from 25 subjects, thus no corrections for repeated 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.

Lichen sclerosis 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 of biopsy frequency in basal cell carcinoma.³¹ 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 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 pre-set of characteristics scored in this analysis, but could be considered for follow-up studies.²⁶ 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.

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 was distinctively identified in LS.

Table 1 Overview of selected morphological RCM characteristics based on literature.

Characteristic	Example	Scoring	Associated Diseases	Description	
Epidermis	Normal honeycomb	1A	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	1B	Absent/ Present	vHSIL, LS, actinic keratosis, invasive squamous cell carcinoma, 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	1C	Absent/ Present	LS, keratosing diseases (actinic keratosis, seborrheic keratosis), squamous cell carcinoma, warts, 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	1D	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	1E	Absent/ Present	vHSIL, LS	Disappearance of the dermal papillae structure and thickening or degeneration of the basal layer has been described in lichen sclerosus. ²⁴
	Dermal sclerosis	1F	Absent/ Present	Lichen sclerosus, lupus erythematosus	Thick and increased number of dermal fibers. Dermal sclerosis is a key feature of lichen sclerosus, also described in histological assessments. ^{14,25}
Immune infiltrate	Lymphocytic exocytosis	1G	Absent/ Present	vHSIL, LS	Single or aggregates of round-to-polygonal, mildly refractive cells at the level of the stratum spinosum interspersed between keratinocytes. ¹⁴ Lymphocytes in the epithelium have been described in histological assessments of vulvar disease, indicating immune responses. ^{25,26}
	Lymphocyte infiltrate	1H	Absent/ Mild/ Moderate/ Profound	vHSIL, LS, 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 1F different type of immune cells. ^{25,26}
	Melanophages	1I	Absent/ Present	Chronic inflammatory diseases, e.g. vHSIL, 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}

(Continuation Table 1)

Characteristic	Example	Scoring	Associated Diseases	Description
Vessels	Perivascular infiltrate	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 sclerosis. ^{25,26}
	Ectatic vessels / capillaries in dermal papillae	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 sclerosis. ^{25,26}
	Hyalinised or sclerotic vessels	Absent/ Mild/ Moderate/ Profound	LS	Commonly described in histological assessment of lichen sclerosis. ^{25,26}

HV=healthy volunteer, vHSIL=vulvar high grade squamous intraepithelial lesion, LS=lichen sclerosis, DEJ=dermal-epidermal junction. volunteer. RCM = reflectance confocal microscopy

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 papillar 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 melanophages in 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) Ectatic 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 on opposite page)

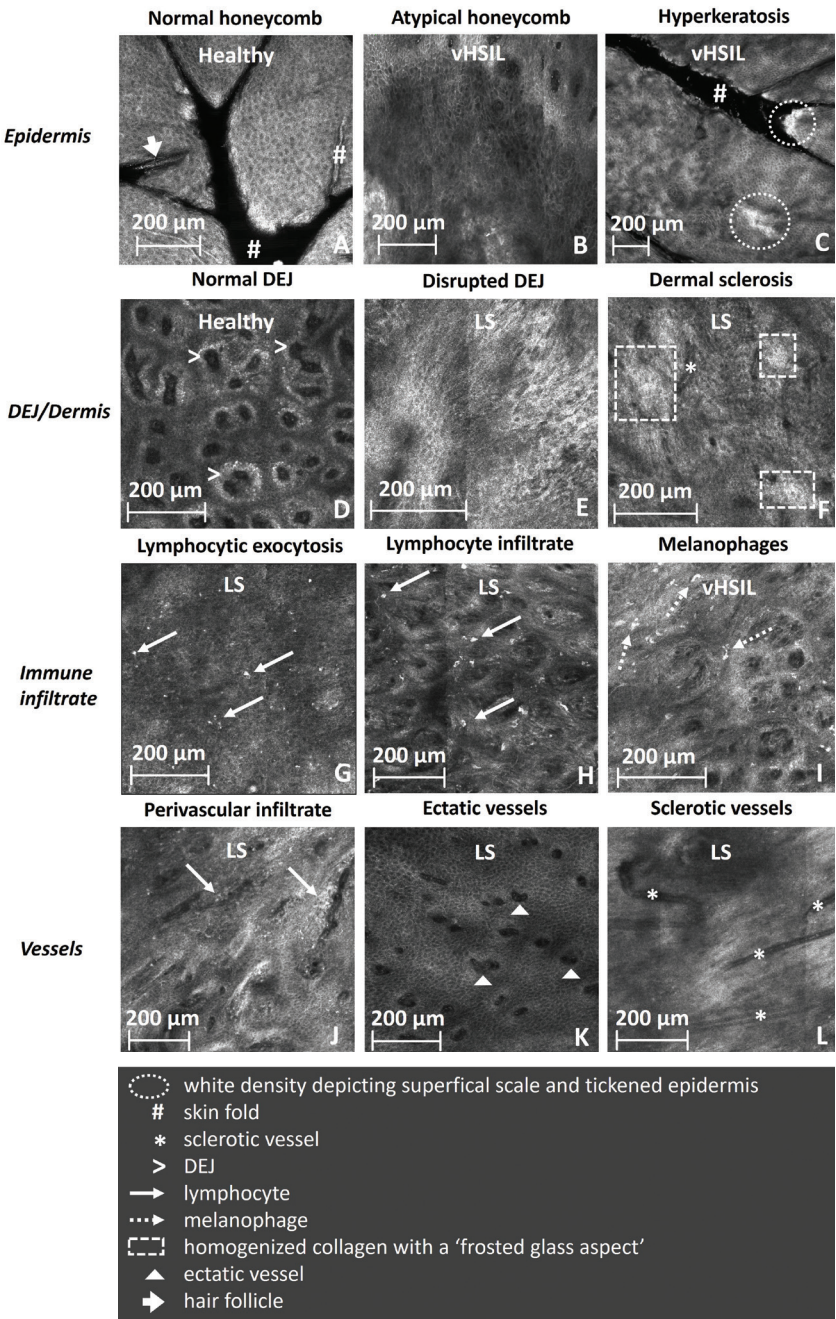
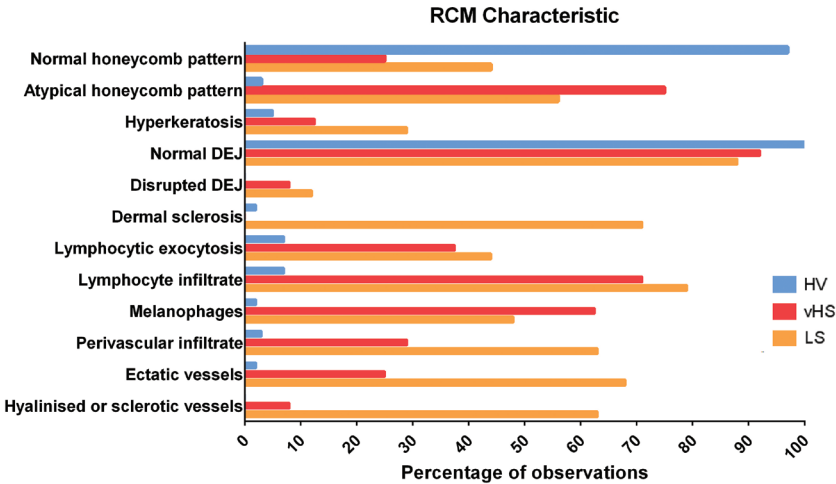


Figure 2 Percentage observed characteristics in RCM images, in HV, vHSIL and LS. The average percentage of observations of both two raters were shown.



HV=healthy volunteer, n=58 observations, vHSIL=vulvar HSIL, n=24 observations, LS=Lichen sclerosus, n=84 observations. DEJ=Dermal Epidermal Junction.

Figure 3 Images of representative lesional skin of vHSIL in the peri-anal area.

A) H&E staining of a 4 mm punch biopsy of lesional warty-type vHSIL on the perineum. Scale bar represents 200 μ m. 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 H&E staining as annotated in A. Dashed areas 1,2,3 and 4 represent skin layers stratum corneum, stratum granulosum, stratum spinosum and epidermal-dermal junction (EDJ) respectively, matching to the layers shown in RCM images D-G. Scale bar represents 200 μ m. D) RCM image of the stratum corneum with skin folds (# signs) (depth 0 μ m). E) RCM image of the epidermis showing an atypical honeycomb pattern and lymphocyte influx with melanophages (white arrows) (depth 41 μ m). F) RCM image showing the epidermal-dermal junction (> sign) (depth 81 μ m). G) RCM image showing the dermis and the dermal papillae with the epidermal-dermal junction (> sign) (depth 121 μ m). 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 μ m.

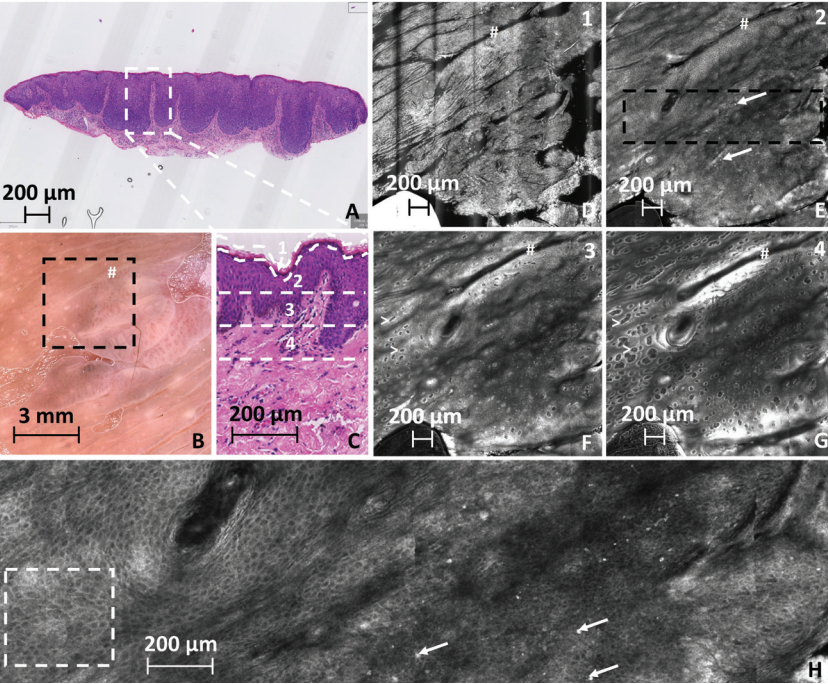
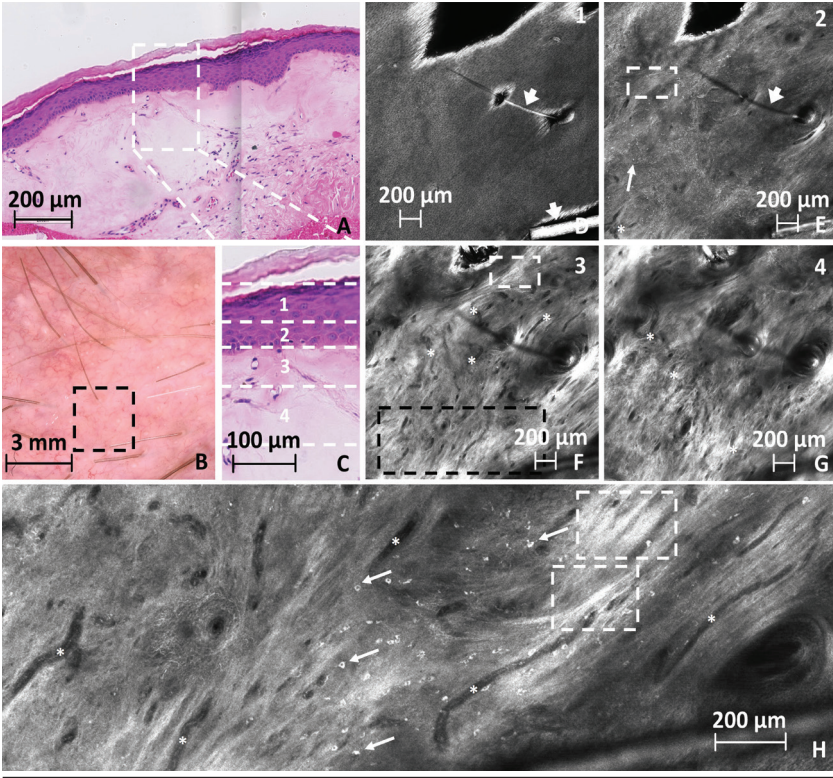


Figure 4 Images of representative vulvar skin of a LS patient – labia majora inner side. A) H&E staining of 4 mm punch biopsy of lesional LS skin on the inner side of the labia majora. Scale bar represents 200 μ m. B) Macroscopic dermatoscopic image of corresponding lesional biopsy (LB) location with black insert of the close-up of the RCM images in D-G. Scale bar 3 mm. C) Insert of H&E staining represented in A. Dashed areas 1,2,3 and 4 represent skin layers stratum corneum, stratum granulosum, 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 μ m. D) RCM image at the stratum corneum with a visible hair (arrowhead) (depth 29 μ m). 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 μ m). F) RCM image showing the dermis with pronounced sclerosis (white dashed box) and hyalinised, sclerotic vessels (asterixes) (depth 126 μ m). G) RCM image showing the deep dermis with distinct sclerosis (white dashed box) and hyalinised vessels (asterixes) (depth 175 μ m). 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 μ m.



SUPPLEMENTS CHAPTER 4

Supplemental data can be accessed online at:

Supplementary Figure 1	In vivo VivaScope 1500/3000 system
Supplementary Figure 2	Mean (SD) patient rating of patient acceptability
Supplementary Figure 3	Images of representative healthy vulvar skin on the labia majora
Supplementary Figure 4	Percentage observed characteristics in RCM images, in non-lesional LS
Supplementary Table 1	Baseline characteristics



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