Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

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Dedicated to my son Dimitrios and his generation.

“I know that I know nothing”

– Socrates (Greek philosopher, 470-399 BC)
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AUTHOR
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Abstract

Histopathologic examination of tissue biopsies is the current gold standard for the diagnosis of dermatological problems. Similarly, in oncology, sentinel lymph node (SLN) biopsy is the state-of-the-art diagnostic method for metastasis screening. Although these methods are safe, they are associated with some limitations, particularly because they are invasive, labor-intensive, and time-consuming. Moreover, histopathological analysis does not always lead to conclusive results. Therefore, there is a need for the development of fast, accurate, and non-invasive diagnostic procedures, complementary to these standard techniques. It seems that laser scanning optical microscopy modalities have the potential to meet this need. Regarding this, the present study was conducted to explore the efficiency of two of these methods, namely reflectance confocal microscopy (RCM) and multiphoton microscopy (MPM), in dermatological and oncological applications. Particular focus was given to lichen sclerosus (LS), basal cell carcinoma (BCC), and malignant melanoma (MM) metastases, all of which are important disorders requiring improved diagnostic methods.

This thesis builds upon the work reported in five papers. The first two papers involved the investigation of LS. In the first paper, we reported the clinical signs of LS, namely hypopigmentation, petechiae, and preputial constriction, based on which the diagnosis of LS was established. In the second paper, it was found that RCM could visualize the thick fiber structures corresponding to sclerosis in the dermis, thereby facilitating the differentiation of LS from normal penile skin. In the third paper, it was observed that the application of methyl-aminolaevulinic acid (MAL) on BCCs could not increase the contrast when imaged with ex vivo MPM. Furthermore, it was found that MAL-induced fluorescence cannot be excited by the expected two-photon excitation route when studied with MPM; rather, a one-photon process (i.e., anti-Stokes fluorescence) takes place. This finding is important not only for diagnostic aspects but also for future work in which photodynamic effects might be required. The fourth and fifth papers involved the investigation of MM metastases in lymph node tissues. It was found that particularly ex vivo
MPM can enable the visualization of the characteristic morphologic features in this type of tissue. Furthermore, by extending the spectroscopic information to include also fluorescence life-time, the latter has the potential to increase the diagnostic ability.

Taken together, the obtained results were indicative of the potential of laser scanning microscopy techniques as diagnostic tools for the detection of LS, BCCs, and MM metastases to lymph nodes. Future studies are encouraged to fully explore the potential of these techniques to be used for dermatological and oncological investigations in a non-invasive/intravital manner.
List of publications

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR

Despoina Kantere
The work presented in this thesis is based on five research articles referred to as Paper I-V.

**PAPER I**
The clinical spectrum of lichen sclerosus in male patients—a retrospective study.
Despina Kantere, Gun-Britt Löwhagen, Gunilla Alvengren, Anna Månesköld, Martin Gillstedt and Petra Tunbäck
Acta Derm Venereol 2014; 94: 542-546

**PAPER II**
Exploring laser scanning microscopy as a non-invasive diagnostic tool for genital lichen sclerosus.
Despoina Kantere, Noora Neittaanmäki, Kristina Maltese, Ann-Marie Wennberg Larkö, Marica B. Ericson, Petra Tunbäck
In manuscript

**PAPER III**
Anti-Stokes fluorescence from endogenously formed protoporphyrin IX – Implications for clinical multiphoton diagnostics.
Despina Kantere, Stina Guldbrand, John Paoli, Mattias Goksör, Dag Hanstorp, Ann-Marie Wennberg Larkö, Maria Smedh, and Marica B. Ericson.

**PAPER IV**
Label-free laser scanning microscopy targeting sentinel lymph node diagnostics – A feasibility study ex vivo.
Despoina Kantere, Jan Siarov, Shahin De Lara, Samad Parhizkar, Roger Olofsson Bagge, Ann-Marie Wennberg Larkö, and Marica B. Ericson
Translational Biophotonics. 2020; https://doi.org/10.1002/tbio.202000002
PAPER V:
Report on fluorescence lifetime imaging using multiphoton laser scanning microscopy targeting sentinel lymph node diagnostics.
Jeemol James, Despoina Kantere, Jonas Enger, Jan Siarov, Ann-Marie Wennberg Larkö and Marica B. Ericson

RELATED PUBLICATIONS NOT INCLUDED IN THIS THESIS:
Clinical Features, Complications and Autoimmunity in Male Lichen Sclerosus.
Despina Kantere, Gunilla Alvegren, Martin Gillstedt, Fani Pujol-Calderón and Petra Tunbäck
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**AUTHOR**
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CHAPTER 1
Preface

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

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The book you are holding in your hand is the summary of a very interesting research I have been performing over the last ten years at the Department of Dermatology and Venereology, at the Sahlgrenska University Hospital in collaboration with the Biomedical Photonics Group, at the Department of Chemistry and Molecular Biology at the University of Gothenburg, Sweden.

But let’s start the story from the beginning. I had the incredible opportunity to be offered a position as a resident at the Department of Dermatology and Venereology at the Sahlgrenska University Hospital. Ann-Marie Wennberg, who is also my main supervisor, played a key role in embarking on this dissertation journey. Not only did she hire me in the department, but she also gave me the honor of joining the research team at the clinic. Within a short time, I had the chance of receiving two simultaneous offers for two different projects. I owe one of these opportunities to my second supervisor, Petra Tunbäck. At the time, Petra was enthusiastically searching for a partner to conduct research on genital lichen sclerosus in men, given the scarcity of reports at that time about the presentation of lichen sclerosus and its possible correlation with penile cancer. Needless to say, I immediately accepted the very interesting offer. Almost at the same time, I was suggested another great opportunity to continue a research project carried out by my colleague John Paoli and my third supervisor, Marica Ericson. For the first time, they had published the very promising results regarding the application of label-free multiphoton microscopy for the diagnosis of basal cell carcinoma. However, the replacement of the established histological analysis with this technique certainly requires further research.

The ideas for the next three projects came up in a very natural way. Researchers usually face new questions throughout the process of performing research. In order to find the answers to the newly generated questions, they opt for launching new research. Therefore, in the third project, we investigated the possibility of replacing histological examination with reflectance confocal microscopy in patients with genital lichen sclerosus with the hope of saving patients from going through the difficult process of the biopsy of the sensitive...
genital area. We also naturally hit on the idea of the fourth and fifth projects because as dermatologists, we want to always attempt to detect melanoma at its early stages to make the best prognosis for our patients. The progression of melanoma to the lymph nodes largely determines the prognosis and treatment choice. Therefore, we decided to examine the potential of reflectance confocal microscopy and multiphoton microscopy for the detection of metastases in an accurate, sensitive, and less invasive manner.

It took many years to carry out and publish the above-mentioned projects. Over years, I reflected a lot. During the long hours of microscopy, I often thought about the reasons that led us to perform research. Probably, the driving force for us as doctors is the same as the one that pushed us toward studying medicine. We became doctors to diagnose different diseases, preferably at an early stage, to be able to fight them better. This task is difficult and arduous. Every time, we meet a patient with a problem, we think somewhere deep inside and wish there was this magical tool that allowed us to make a diagnosis without any delay or high expense, easily and painlessly, and above all, with great accuracy. The achievement of this end would accelerate the determination of the treatment approaches. Of course, after these enchanting dreams, unfortunately, we realize that this magical tool does not exist at the moment. However, the thoughts are then refreshed again, and we tend to muse that this machine may exist in the future if we build it little by little. However, in this multifaceted, long journey of research, this beautiful but ambitious goal seems to be elusive and somewhere very far away, which may weaken over time and wear away in the end. However, the moment you lose, hope arrives. And then, once again, it can get even harder when you think that everything you do makes no sense. It was the time I realized that the history of each project separately and all projects together is not exactly like the fantastic and exciting stories in which everything is solved magically and without much explanation, where one usually presses a button, and the problem is solved. In fact, in the history of research, nothing is magical; you struggle to prove what you believe, and the results can lead you to a maze of thoughts or even to a stifling impasse. And at best, what you did was opening a door that hid more labyrinths, with the truth being always hard to find and reveal. Personally, during a great proportion of this trip, I continued to like the fantasy stories, in which everything is solved magically. Because the research stories are difficult, you start and build them; however, they may collapse because you didn’t put a piece well from the beginning and then you need to rebuild them. Accordingly, it is not easy to tame these stories, direct them, process them, and finally write them
down because they only like the squares of their logic, which nourish them. As can be inferred, for many years, imaginary stories have been my favorite, and my research stories have always been a challenge. But it is this challenge that made them tempting.

Year after year and experiment after experiment, my curiosity grew more and more to see what would happen at the end of this story. Toward this end, I was looking forward to its release as I did with every other interesting, fantastic story generated from intrigue and mystery. But I will not tire you anymore. What happened finally to these research stories, these five dissertation projects? Read the book below, and you will learn what happened in the end. Oh, and don't forget the epilogue.
CHAPTER 2
Abbreviations

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
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## Abbreviations

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<th>Description</th>
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<td>Aminolaevulinic acid</td>
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<td>BCC</td>
<td>Basal cell carcinoma</td>
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<td>FLIM</td>
<td>Fluorescence lifetime imaging</td>
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<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<td>LS</td>
<td>Lichen sclerosus</td>
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<td>LN</td>
<td>Lymph node</td>
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<td>MAL</td>
<td>Methyl-aminolaevulinic acid</td>
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<td>MM</td>
<td>Malignant melanoma</td>
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<td>MPM</td>
<td>Multiphoton microscopy</td>
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<td>PpIX</td>
<td>Protoporphyrin IX</td>
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<td>RCM</td>
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<td>SLN</td>
<td>Sentinel lymph node</td>
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CHAPTER 3
Introduction

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
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Introduction

Diagnosis is referred to as the art or act of identifying a disease based on its signs and symptoms. A precise diagnosis facilitates the achievement of positive health outcomes because clinical decisions and treatment will be tailored to the correct comprehension of the medical issue [1]. The diagnostic process consists of acquiring clinical history by interviewing, performing a physical examination, and using diagnostic tests. The main forms of diagnostic tests are laboratory medicine and medical imaging.

Nowadays, the advancement of imaging technologies has helped physicians to detect, diagnose, and treat conditions in a better, non-invasive manner. However, the implementation of biopsy as an invasive procedure is inevitable in many dermatological disorders. The histopathologic analysis of skin biopsies is considered a gold standard for diagnosis. Lichen sclerosus (LS) and basal cell carcinoma (BCC) are two of the skin disorders the confirmatory diagnosis of which often requires a biopsy. Although the procedure of obtaining a skin biopsy is generally considered safe, it can potentially lead to the development of such complications as bleeding, infection, and aesthetic problems (e.g., scarring, hyperpigmentation, and hypopigmentation, especially in sensitive body areas, such as the face and genital). Moreover, histopathological analysis does not always lead to conclusive results.

Similarly, in oncology, sentinel lymph node (SLN) biopsy is an invasive procedure designed to stage malignancies, such as malignant melanoma. Despite the significant contribution of SLN biopsy in improving staging in patients with melanoma [2], this technique has some limitations, primarily associated with histopathological analysis, which is known to be labor-intensive and time-consuming [3]. Nevertheless, extensive pathological workup can reduce the false-negative rate of this method [4] but at the cost of higher laboratory workload, resulting in prolonged responses.

Therefore, there is a need for a fast, accurate, and non-invasive diagnostic procedure for dermatological problems. Likewise, with regard to oncology, considering that early diagnosis and metastasis staging are crucial in cancer management, novel technologies are required to rapidly facilitate the
assessment of tumor progression. A number of different optical methods have been developed in the last decades with the aim of improving the diagnostic procedures.

The study of laser scanning microscopy techniques was one of the main targets of the current thesis. Two kinds of these methods are in vivo reflectance confocal microscopy (RCM) and ex vivo multiphoton microscopy (MPM). Both of these techniques operate in the near-infrared (NIR) wavelength region, which has a higher penetration depth in the tissue. The RCM operates by detecting the backscattered light, using a continuous wave NIR laser in a confocal setting. These techniques have already been translated into clinical practice in the field of dermatology [5-9]. More specifically, RCM has been verified for the clinical diagnosis of skin cancer [5], while MPM has the potential to be used for the histopathological analysis of tissues without extensive tissue workup [10-13].

With this background in mind, the aim of this thesis was to investigate the potential of RCM and MPM in identifying the characteristic morphological features of LS, BCC, and MM metastatic to lymph nodes in order to improve the diagnosis of these conditions.
CHAPTER 4
Background

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

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HUMAN SKIN

The skin, consisting of the epidermis and dermis, varies in thickness depending on anatomic location, gender, and age of the individual. This organ has the highest thickness on the palms and soles of the feet, while its thinnest part is found on the eyelids and in the postauricular region \[14\]. The epidermal layer of the prepuce and glans is approximately 140 μm \[15,16\]. Four types of cells, namely keratinocytes, dendritic cells, melanocytes, and Merkel cells, are found within the epidermis. The epidermal-dermal junction has a characteristic, undulating rete ridge structure rather than a flat surface. The dermis primarily consists of extracellular matrix tissue, including collagen and elastin fibers, as well as a sparse population of cells, including fibroblasts, macrophages, and adipocytes. The vasculature and lymphatic vessels can be also found within the dermis \[17\].

LICHEN SCLEROSUS

Lichen sclerosus (LS) is a chronic inflammatory disorder of the anogenital area, occurring in both males and females. The prevalence of LS varies from 0.0014% to over 0.01% in adult men; however, it may be underreported \[18\]. The underlying cause of this disorder is unknown; nonetheless, there are studies introducing genetic predisposition and underlying autoimmune mechanisms as important predisposing factors \[19\]. Furthermore, LS has been associated with the alteration of methylation patterns and gene expression in the affected tissues \[20,21\]. The changes in gene expression and chronic inflammation may untreated LS a risk factor for the squamous cell carcinoma (SCC) of the genital area \[22\].

The LS in men can involve the prepuce, glans penis, and meatus. It often presents with hypopigmented areas, petechiae, preputial constriction, and meatus stricture as exemplified by Figure 1. Furthermore, buried/trapped penis and recurrent skin infections are typical of LS in obese patients \[23\]. The diagnosis of LS is commonly confirmed with a skin biopsy. This technique
is also used to confirm or rule out cell dysplasia in LS lesions. However, the biopsy procedure is accompanied by some complications, such as pain and bleeding in the sensitive genital area, as well as scars and aesthetic problems.

The characteristic histopathological features of LS are thinning of the epidermis, band-like infiltrate of lymphocytes and plasma cells in the dermis, and hyalinization of the collagen located in the upper dermis. Moreover, the degeneration of the basal cell layer can also be seen. Other histopathological features that can be found in LS are orthokeratosis, hyperkeratosis, spongiosis, pigmentary incontinence, and follicular plugs [17].

In addition to itch and pain, LS can significantly affect the sexual life of the patient and cause moderate to severe psychological problems [24], thereby diminishing the quality of life of the affected patients. Genital LS increases the risk of developing penile intraepithelial neoplasia (PeIN) as well as penile SCC [25]. The LS may represent the initial step in a non-HPV-related oncogenic pathway for SCC [26, 27] or act as a field susceptible to HPV-related dysplastic

Figure 1. Lichen sclerosus with preputial constriction and hypopigmented areas.
changes\textsuperscript{[28]}. Moreover, there is a body of evidence on the incidence of similar epigenetic alterations, such as the hypermethylation of specific genes, in LS and penile SCC, suggesting that these play a role in the transition from benign to malignant lesions\textsuperscript{[21,29]}. The evidence is confirmative of the association between SCC and LS in females. Nevertheless, the correlation between penile cancer and LS is still unclear. Regarding this, the present thesis was also targeted toward investigating the risk of developing penile cancer in males with LS.

**SKIN CANCER**

**Basal cell carcinoma**

Basal cell carcinoma (BCC) is an epithelial neoplasm of the skin, which is recognized as the most common human malignancy. This disease most often occurs in the sun-exposed areas of the skin. The rate of BCC is much higher in the fair-skinned individuals than in the dark-skinned ones\textsuperscript{[30]}. The incidence of BCC is on a continuously increasing trend worldwide. More specifically in Sweden, the number of people developing BCC in one year is reported to range within 30,000-35,000\textsuperscript{[31]}.

The BCC grows slowly and metastasizes in rare cases. The morphological classification of BCC includes superficial, nodular (with micronodular), infiltrative (with morphoeic), and mixed subtypes. The present thesis involved the investigation of superficial BCCs. The latter presents clinically as an erythematous, well-circumscribed macule with a minimal scale. There is often a central clearing and a threadlike border. The BCCs spread horizontally invading the surrounding tissues.

The diagnosis of superficial BCC is primarily performed clinically based on the above-mentioned description and the presence of typical dermoscopic features, such as shiny, white to red areas, erosions, and short fine telangiectasias\textsuperscript{[32]}. However, it is often confirmed histopathologically by obtaining a skin biopsy. The histological presentation of this skin cancer is characterized by numerous, small, basaloid nests attached to the lower part of the epidermis, separated by normal epidermis. The cells have round or oval basophilic nuclei that are larger and darker than those of the epidermal basal keratinocytes; furthermore, they have a minimal cytoplasm\textsuperscript{[33]}. However, the skin biopsy procedure can potentially lead to such complications as bleeding, infection, and aesthetic problems (e.g., scarring, hyperpigmentation, and hypopigmentation), especially in sensitive body areas, such as the face.
Malignant melanoma and Sentinel lymph node biopsy

Malignant melanoma is a neoplasm of melanocytes that is considered one of the most aggressive form of skin cancer (Figure 2). It is the fifth most common cancer in both genders [34]. The outcome of melanoma depends on the stage of the disease at the time of diagnosis. The MM often metastasizes through lymphatic channels to regional lymph nodes. Accurate staging upon diagnosis is important to assess the prognosis of this disease and determine the need for adjuvant therapy and patient eligibility for entering clinical trials.

The SLN biopsy is used in the staging of MM, based on the fact that the cutaneous site of the melanoma drains into one or two sentinel nodes (but rarely more lymph nodes), which are the first sites of metastasis. The identification

Figure 2. Image of a malignant melanoma with irregular borders, multiple colors and asymmetrical distributed pigment.
and marking of the draining lymph node basins for a given melanoma site are performed using lymphoscintigraphy. Typically, technetium sulfur colloid and blue dye are intraoperatively injected into the skin surrounding the site of the excised melanoma. The identification of the “blue or hot” sentinel nodes is accomplished with the aid of a hand-held gamma counter and visual inspection. These nodes are selectively biopsied and then subjected to further examination by serial H&E stained sectioning complemented with immunohistochemistry (S100, HMB45).

In case of the diagnosis of melanoma metastasis, a complete regional lymph node removal is normally performed. The results of the Multicenter Selective Lymphadenectomy Trial-I confirmed the role of lymphatic mapping with SLN biopsy as a prognostic tool \[^{[35]}\]. In the mentioned trial, patients with intermediate-thickness melanoma and microscopic lymph node involvement, who were subjected to lymphatic mapping with SLN biopsy and early regional lymphadenectomy, survived for a longer time than those managed with a wide excision of the primary melanoma, followed by observation without SLN biopsy.

The SLN biopsy has a number of limitations, associated with histopathological analysis, known to be labor-intensive and time-consuming. Moreover, this technique reportedly has low sensitivity, with a false-negative rate of up to 20% \[^{[3]}\]. Extensive pathological workup can reduce the false-negative rate of this method \[^{[4]}\], but at the cost of higher workload and prolonged referral responses. In addition, SLN biopsy is an invasive surgical procedure that can lead to unwanted complications, such as seroma (i.e., the accumulation of fluid at the site of surgery), postoperative infection, lymphedema, numbness and postoperative pain, or even impaired wound healing \[^{[36, 37]}\]. Therefore, there is a need for developing new techniques to facilitate the diagnosis of MM metastases in the lymph nodes, which is part of the aim of this thesis.

**LASER SCANNING MICROSCOPY TECHNIQUES FOR IMPROVING DIAGNOSTICS**

The RCM and MPM are both laser scanning microscopy techniques operating in the NIR wavelength region. They are using focused laser beams that scan the investigated tissue via optical sectioning. The resolution of the RCM ranges typically 1.0 μm in the lateral direction and 3 to 5 μm in the axial direction. This modality offers the possibility of detecting cellular structure down to the upper dermis, with an image depth of 150-200 μm. Likewise, MPM, which is a more expensive technique than RCM, operates in a nonlinear process, using two-photon excitation only occurring in the focal plane that enables tissue
autofluorescence. This modality has a slightly better resolution than RCM, which is typically around 0.5 and 1-2 μm in the lateral and axial directions, respectively. A more detailed review of these techniques is provided in the next sections.

**Reflectance confocal microscopy (RCM)**

Reflectance confocal microscopy operates by illuminating a biological tissue, followed by detecting the backscattered light and displaying it on a monitor. Figure 3 displays a picture of a clinical RCM device. The light that is scattered back from the tissue creates contrast due to the different refractive indices of water and tissue constituents [38]. Melanin, melanosomes, keratin, and collagen, found abundantly in the skin, are highly reflective and diffractive of the illuminating light. Consequently, they produce a high contrast (white) against their surrounding molecules (dark). The RCM provides an in vivo horizontal view of the skin. The image depth of the RCM in the skin is 150-200 μm in practice, which, in the normal skin, corresponds to the upper papillary dermis.

![Figure 3. In vivo reflectance confocal microscopy device, located at the Department of Dermatology and Venereology, Sahlgrenska University Hospital, Gothenburg, Sweden.](image)

More specifically, RCM has been introduced as a complementary tool for dermoscopy and clinical examination. This technique is used to diagnose skin malignancies and disorders as a substitute for a skin biopsy [39-41]. In a study, RCM and dermoscopy could detect melanoma with 98% sensitivity [42]. In two other studies, the sensitivity of RCM interpretation for detecting BCC was 100% [43, 44]. Moreover, RCM has been successfully used for defining the surgical margins in Mohs operations for lentigo maligna and melanoma [45, 46]. Furthermore, RCM has been utilized...
for the in vivo investigation of a broad spectrum of both infectious and inflammatory skin disorders, such as psoriasis, eczema, and contact dermatitis [38]. This method may be also useful for monitoring treatment response for skin inflammation in lupus erythematosus, psoriasis, and lichen planus [47].

Consequently, the use of RCM can facilitate the improvement of clinical diagnostic accuracy and reduction of the number of unnecessary biopsies of the skin lesions. Moreover, RCM seems to be a promising tool for diagnosing and monitoring inflammatory lesions. However, due to the lack of adequate guidelines and official protocols in this domain, this imaging modality is not yet broadly applied in clinical practice.

**Multiphoton microscopy (MPM)**

Multiphoton microscopy is a fluorescence imaging technique, the underlying principle of which is based on breaking the fundamental quantum physics laws [8]. In contrast with conventional fluorescence microscopy, MPM is based on non-linear optical signal generation. This technology often utilizes the process of two-photon excitation; however, second-harmonic generation (SHG) is also applicable. In the last decades, MPM has evolved into an important laboratory technique in biosciences [8], facilitating the visualization of biological tissues in a three-dimensional, non-invasive manner. Figure 4 illustrates the picture of a MPM device. The skin structure can be studied with the aid of MPM in a label-free set-

![Figure 4. Multiphoton microscopy device at the Center of Cellular Imaging, University of Gothenburg, Gothenburg, Sweden (Photo: Despoina Kantere)](image-url)
ting by visualizing the skin constituents using their endogenous contrast. For example, keratinocytes can be visualized based on nicotinamide adenine dinucleotide (NADH) and flavine adenine nucleotide (FAD). Keratin can be also visualized by its endogenous fluorescence. Collagen and elastin are observable in the dermis owing to their endogenous fluorescence. In addition, several isoforms of collagen, including type I, often produce SHG signal due to their non-centrosymmetric molecular structure and crystalline organization. Melanin has also been an important target in MPM imaging.

Furthermore, tumor biology is extensively studied with MPM, by imaging tumor-associated blood and lymphatic vessels. Paoli et al. showed that ex vivo MPM could facilitate the visualization of several diagnostic criteria of superficial BCCs by providing autofluorescence data. Moreover, previous research suggested that endogenously formed protoporphyrin IX (PpIX) can be used to improve the skin tumor contrast. The application of MPM has been even extended to immune imaging, with MPM equipment, specifically constructed for this purpose, which enables cell tracking and analysis.

Along with the natural fluorophores generating autofluorescence, external fluorophores (e.g., organic compounds) and genetically expressible fluorescent proteins added to the investigated samples have been extensively used to increase the imaging contrast ex vivo. Nevertheless, there is a need for developing fluorophores that are compatible with life and can be used in an intravital and in vivo imaging. One substance that can be used for this purpose is aminolaevulinic acid (ALA) and methyl aminolevulinate (MAL). When applying ALA and MAL on living tissues, they transform to PpIX through the pathway of heme biosynthesis. The ALA-induced PpIX is accumulated in tumors; accordingly, it is reportedly a tumor marking fluorophore. Taken together, the ability of MPM to visualize autofluorescence and PpIX makes it a promising tool facilitating the improvement of diagnosis.

The technique of two-photon excitation can be combined with other well-established techniques, such as fluorescence lifetime imaging (FLIM), for the investigation of tissue and cell cultures. The FLIM is a method that can be applied for the dynamic measurement of different fluorescence lifetimes by detecting the changes in the binding, conformation, and composition of biological tissues, thereby allowing for the study of the structures and function of the tissues. This is achieved by using the basic property of fluorescence lifetime; the fluorescence lifetime of a fluorophore is primarily dependent on its molecular environment, but less on its concentration. Among the FLIM tech-
niques, the time-correlated single-photon counting delivers the highest time resolution and is perfectly compatible with MPM and confocal laser scanning microscopy [60, 61]. In this thesis, ex vivo MPM and ex vivo experimental MPM-FLIM devices were used. A thorough review of these devices is presented in the method section.
CHAPTER 5
Aims

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy
Aims

The overall aim of this thesis is to study the application and potential of the laser scanning microscopy techniques in the diagnosis of LS, BCC, and MM metastases in lymph nodes. More specifically the aims of the reported projects have been as follows:

**PAPER I**
To investigate the clinical features on which the diagnosis of LS is based, the number of cases where a biopsy was taken in order to confirm the diagnosis, and the correlation between LS and penile cancer.

**PAPER II**
To investigate if RCM in vivo can identify morphological features on LS lesions that can be correlated to their histopathological counterparts.

**PAPER III**
To investigate if MAL can be used to increase contrast in BCCs when imaged with MPM ex vivo.

**PAPER IV**
To investigate if MPM and RCM ex vivo can visualize MM lymph node metastases.

**PAPER V**
To investigate if MPM-FLIM ex vivo can distinguish differences between metastasized and non-metastasized SLN tissue.
CHAPTER 6
Methods and materials

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
Despoina Kantere
Methods and materials

STUDY PARTICIPANTS AND TISSUE SAMPLES

PAPER I
Clinical records
Clinical records from male patients diagnosed with LS in the Department of Dermatology and Venereology at the Sahlgrenska University Hospital, in Gothenburg and at the Södra Älvsborgs Hospital, in Borås, in Sweden, during the period 1997–2007 were reviewed. A number of 771 patients were included in the study. The inclusion criteria were valid LS diagnosis based on typical histopathology, or typical clinical features, including hypopigmentation, petechiae and preputial constriction. All study participants were ≥ 18 years.

Swedish Cancer Registry
The cases of penile cancer and PeIN found in the clinical charts of the patients included in the study were correlated with information from the Swedish Cancer Registry regarding all the included patients.

Questionnaire
A questionnaire was sent to all included patients. It contained inquiries about their symptoms related to LS, circumcision, number of appointments with the dermatologist, various treatments that were used, other autoimmune diseases, and the impact of LS on their sexual health.

PAPER II
Participants in the study
The patients were included in the study when visiting the Department of Dermatology and Venereology, at the Sahlgrenska University Hospital, in Gothenburg, Sweden, from 2018 to 2020. The inclusion criteria were ≥18 years of age, histopathologically confirmed LS and no use of topical treatment 14 days prior to the inclusion in the study. In total 30 patients were included in the
study, of which 16 patients were diagnosed with LS. As controls four healthy individuals, six patients with nonspecific balanoposthitis, three with plasma cell balanitis and one with psoriasis were included. 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. The individuals with healthy genital skin were asymptomatic patients visiting the clinic in order to exclude sexually transmitted diseases, as well as patients who were evaluated for extragenital skin disorders.

**Questionnaire**

The patients diagnosed with LS answered a questionnaire that contained inquiries related to LS, circumcision, treatment, and experiences from the biopsy procedure.

**PAPER III**

A total of 12 patients were included in the study when visiting the Department of Dermatology and Venereology, at the Sahlgrenska University Hospital, in Gothenburg, Sweden, from 2008 to 2012. All the patients had histopathologically verified superficial BCCs. The biopsies obtained from the BCCs of the first nine patients were imaged with MPM ex vivo using 780 nm excitation; however, no difference between the autofluorescence and the PpIX channel was observed. The data of these measurements are not presented in the published paper. Nevertheless, three more patients with superficial BCC were included in the study. The data of these three patients are presented in the published paper.

MAL cream (METVIXâ, Galderma, 160 mg/g methyl aminolevulinate as hydrochloride) was applied on the BCCs of two patients for 3 hours. Placebo cream (Unguentum M, Hermal, Rein-bek, Germany) was applied on one BCC in the same manner. The application areas were covered with an occlusive and light-protective dressing.

The BCCs were removed surgically. Biopsies from both the tumor bulk and perilesional normal skin were obtained. The biopsies were trimmed with surgical scissors, eliminating a part of subcutaneous tissue. Half of the tissue biopsy obtained from one of the MAL treated patients were cryosectioned in 10 mm thin tissue sections and mounted on microscope slides, while the other half were stored in a freezer (-70 °C). The full thickness skin biopsies were placed in an imaging chamber gasket with the skin surface against the cover glass and mounted onto a microscope slide. The slides with the prepared specimens were
PAPER IV AND PAPER V

Twelve lymph node samples were randomly obtained from a biobank at the Department of Pathology, at the Sahlgrenska University Hospital, in Gothenburg, Sweden, holding 215 excised lymph node tissues from all patients with melanoma undergoing SLN surgery at the hospital during the years 2012–2015. The tissue blocks, together with corresponding hematoxylin and eosin (H&E) stained slides, were analyzed and then returned to the biobank within three weeks. Eight of the samples had metastasis and four were negative, serving as controls. One deparaffinized sample with metastases was excluded from the study due to artefacts caused during sample processing. LN1, LN2, LN3, LN4, LN5, LN7, LN8 were investigated with MPM. LN11, LN12 were investigated with FLIM-PM. LN6, LN9 and LN10 were investigated with both MPM and FLIM-PM.

When commencing the study, imaging was done on the first four lymph nodes directly on the paraffin-embedded tissue blocks. However, to prevent misinterpretation and the risk of monitoring artefacts, the rest of the tissue samples were deparaffinized and rehydrated before imaging procedure according to a customized protocol.

The samples were mounted in custom made imaging gaskets, as illustrated by Figure 5, with ultrasound gel (LN1, LN2, LN4, LN5, LN6, LN7, LN8, LN10, LN11 and LN12) or without correction (LN3, LN9). The differences in sample mounting techniques were made due to practical reasons and were not found to affect the morphological features observed to any major extent.

Figure 5. Human lymph nodes mounted with ultrasound transmission gel in a microscope slide.
LASER SCANNING MICROSCOPY TECHNIQUES

Reflectance confocal microscopy

In papers II and IV, an in vivo VivaScope 1500™ (MAVIG GmbH, Germany), approved for clinical imaging, was used. The RCM is equipped with a continuous-wave laser operating at 830 nm. The instrument was equipped with a customized objective lens (P/N 04288, NA=0.9, Photon Gear, US), resulting in optical resolution corresponding to 1 µm lateral and 3 µm axial. A standardized image-capturing process was applied in each investigation. Both Vivaslacks in depth of 200 µm and Vivablocks up to 8 x 8 mm were obtained. In Paper II, RCM was used in vivo directly on the patients. Oil was applied to an adhesive window attached to a stainless-steel tissue ring. The window was placed onto the skin area affected by LS or the lymph nodes. Ultrasound gel was applied to the center of the adhesive window. Then, the laser tube of the RCM was affixed to the tissue ring. In Paper IV, RCM was adapted to investigate the lymph nodes ex vivo by placing the lymph nodes on the adhesive window using ultrasound transmission gel to enable optical contact.

Multiphoton microscopy

In this thesis, three different MPM set-ups have been utilized, described as below:

PAPER III

In the initial phase of the study a BioRad Radiance 2100MP Rainbow (Bio-RAD, Hemel Hempstead, UK; company purchased by Carl Zeiss in 2004) was used. The excitation source was a mode-locked, femtosecond pulsed Tsunami Ti:sapphire laser, pumped by a Millenia Xa 10W diode-pump laser. This system was then replaced by a Zeiss LSM 710 NLO system (Carl Zeiss MicroImaging GmbH, Germany), equipped with a Mai Tai HP DeepSee™ (80 MHz, Spectra-Physics, Newport Corporation, USA) tunable in the wavelength range of 690-1040nm. The upgraded MPM system is equipped with a 34-channel QUASAR detection unit making it possible to select specific spectral regions without being restricted to certain filters. This feature was found crucial in order to be able to acquire relevant data for the study. The objective lens used was a 20x water immersion (Zeiss W Plan-Apochromat 20x/10) corrected for coverslips with a thickness of 0.17 mm. The optical resolution was ~0.5 µm lateral and ~1.5 µm axial. The working distance was 1.7 mm. The desired power
at the sample is controlled by an AOM and chosen as percent of transmission in the software. Powers ranging between 30–100 mW were used.

**PAPER IV**

In this paper, the same LSM 710 NLO microscope as above was used for MPM imaging; however, two different tunable fs pulsed NIR lasers were utilized for excitation. The first four tissue samples were imaged using the Mai Tai HP DeepSee” (as above), while InSight X3 (Spectra-Physics, Newport Corporation, USA) was used for the rest of the tissues. The excitation wavelength for both the lasers was set to 780 nm to target autofluorescence, and the power setting was optimized to yield a similar fluorescence signal. Two different Plano-Apochromatic 20x/1.0 (WD 1.9 mm) water immersion objective lenses were used, either with correction for 0.17 mm cover glass on samples mounted with ultrasound or without correction. The fluorescence from the tissue was collected in the emission range of 410–690 nm using a non-descanned, highly sensitive GaAsP detector.

**PAPER V**

An experimental MPM set-up was used for FLIM (see Figure 1 in the published Paper V). Excitation was obtained by an fs-pulsed (~80 fs) tunable (700-900 nm) Ti: Sapphire laser (Tsunami, Spectra physics), pumped by a frequency-doubled Nd: YAG laser (Millenia, Spectra Physics, 532 nm). The output laser power is modulated by a Pockels cell (350-80LA, ConOptics). FLIM was performed using an excitation wavelength of 780 nm, an average laser power less than 15 mW, and 100 fs pulse duration at the sample plane maintained by applying autocorrelator (CARPE, APE) and pulse compressor (Femto control kit, APE). The emission signal was detected by two GaAsP detectors (H7422P-40 MOD, Hamamatsu), interfaced to time-correlated single-photon counting modules SPC 150 (TCSPC, Becker&Hickl) allowing for life-time measurements. Two spectral detection channels were set up using a dichroic mirror (550 nm cut off, Semrock Inc) combined with two filters 580/150 nm and 525/50 nm (Semrock, Brightline), enabling the two channels: 1 (red, 550–655 nm) and 2 (green, 500–550 nm). The FLIM images were acquired at an area of 512 x 512 pixels, using pixel dwell time 1.8 μs and by scanning a frame at a speed of 0.616 sec. Total acquisition time for each image was less than 60 secs. The FLIM analysis was performed using SPCImage 8 software.
(SPCM, Becker&Hickl). Single exponential decay model was applied to fit the fluorescence decays. Fluorescence lifetime data from different regions of interest were extracted from SPCImage and fluorescence decay curve and lifetime histogram were plotted in MATLAB (MathWorks Inc.).

**IMAGE ANALYSIS**

The RCM images were acquired using the VivaScan software and exported as TIFF interface, using mD4 (MAVIG GmbH, Germany). The MPM images were acquired and exported as TIFF, using ZEN software (Carl Zeiss MicroImaging GmbH, Germany). All the images were viewed, and brightness was adjusted using Photoshop (Adobe Systems Inc., USA) for contrast enhancement.

**STATISTICAL ANALYSIS**

Statistical analysis was performed in Paper I using R version 2.14.2, R Foundation for Statistical Computing, Vienna, Austria. Differences in proportions were tested using Fisher’s exact test (P value was calculated exactly). Two sample tests were carried out using Wilcoxon’s rank sum test.

Paper II, III, IV and V were proof-of-concept, descriptive studies, exploring the potential of RCM and MPM to identify morphologic features characteristic for histopathologic diagnosis of LS, BCC and MM metastases. To gain statistical power was beyond the scope of these studies, thus no statistical analysis was performed.
CHAPTER 7
Ethical considerations

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
Despoina Kantere
In accordance with the research ethical principles, all study participants included in the studies signed the informed consent form, and institutional rules for the clinical investigation of human subjects were followed. All the studies were approved by the Regional Ethical Review Board of Gothenburg. The diary numbers are the following:

**Paper I:** 145–08  
**Paper II:** 415–17  
**Paper III:** 229–09  
**Paper IV and Paper V:** 145–16

In Paper I, the main ethical consideration was that reminding the study participants, who received the questionnaire, of their genital disorder could induce awkward feelings in them. To avoid this risk, all patients were offered an appointment at the Department of Dermatology if they wished to discuss their concerns and symptoms.

During the inclusion of the participants in our second study (Paper II), it was very hard to convince healthy patients to be examined with RCM; consequently, a low number of healthy individuals could be included. On the other hand, the patients with LS were quite willing to participate in the study hoping that they could contribute to the better management of LS for themselves and others.

In our third study (Paper III), all the patients included in the research had superficial BCCs. The first-line treatment of superficial BCCs at the Department of Dermatology and Venereology is cryotherapy or electrocautery. However, the study patients were treated instead with the surgical removal of the lesions without any complications.

In papers IV and V, the investigation of the biological specimen (e.g., lymph
nodes) was performed ex vivo after the routine histopathological evaluation was completed by experienced pathologists at the Sahlgrenska University Hospital. Therefore, there was no risk of direct complications for the patients included in the study. However, during the study, the lymph nodes disclosed by the biobank underwent de- and re-paraffinization. This process, along with sample preparation and MPM imaging, could have altered and photodamaged the tissue, thereby adversely affecting the possible future systematic reevaluation of the specimen and its utilization for future studies. It should be noted that no tissue damage was caused during the experiments.

Furthermore, the additional analysis of the tissue with MPM and RCM could hypothetically reveal micro-metastases that were not detected in the conventional histopathological analysis. This could cause psychological distress in patients. Nevertheless, the results of the imaging of the lymph nodes were in total agreement with those of the histopathological diagnosis.

During planning and designing the study, we came across an unresolved, ethical problem. Some of the included lymph nodes belonged to people who were deceased. To overcome this problem, we wrote to the local ethics committee asking them for permission from the relatives of the participants. However, the committee did not grant permission to contact their relatives and asked us to continue our investigation without their permission. However, this requires thorough consideration. Do we really have the right to do so? I am not sure and unfortunately, I cannot ask the deceased people either.
CHAPTER 8
Results, discussion and methodological considerations

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
Despoina Kantere
Results, discussion and methodological considerations

PAPER I

The diagnosis of LS is often made based on typical clinical criteria. In this retrospective analysis, we reported three typical clinical signs of LS; namely hypopigmentation, petechiae, and preputial constriction, on which the diagnosis was established. The clinical signs of LS are often diagnostic. However, when the clinical picture is unclear or the physician is unfamiliar with the disease, a skin biopsy from a lesion is performed. Moreover, this procedure is especially imperative and promptly performed when penile cancer is suspected. In our investigation, biopsies were performed on 35% (273/771) of the included patients, and the histology was classified as compatible with LS in 88% (240/273) of the cases. This high rate is indicative of the importance of this method in LS diagnostics.

Almost 75% (419/557) of the patients did not require no other treatment than a potent steroid. Furthermore, circumcision was already performed on 28% (223/771) of the patients, either upon entering the study or during the research period. Out of these cases, only two patients came back to receive additional therapy with local steroid. The prevalence of autoimmune diseases (e.g., diabetes mellitus, alopecia areata, vitiligo and thyroid disease) in LS patients was compatible with that in the general population.

The prevalence of SCC varies in LS patients; however, this rate has been reported as 1–6 % on average [25,62]. There is also evidence regarding the development of other more uncommon skin cancers such as verrucous carcinoma, BCC, and MM in LS patients [63]. In the present study, 1% (8/771) of the participants developed cancer during the study period. Furthermore, the rate of SCC coincidence was found to be lower in our
study in comparison to the values reported in studies addressing vulval carcinoma in women with LS. However, our participants had a higher rate of penile cancer (0.02%) compared to the whole Swedish male population. This result underlines the need for the follow-up of male patients with LS.

The questionnaires were returned by 59% (456/771) of the patients. According to the answers given in the questionnaire, more than half of the patients noted that the disease had a negative impact on their sexual health, which is in congruence with the results of other studies that reported dyspareunia in 55% of the male patients [64]. Regarding the effect of circumcision on sexual health, 46% of the responders had a better sex life after undergoing the operation. Furthermore, one third of our cohort (142/456) reported to have persistent preputial constriction, and a quarter (124/456) recorded that they had an ongoing/earlier meatal involvement. Notably, about 25% (115/456) of the patients affirmed the achievement of complete recovery, however 26% (30/115) of these cases reported to have residual changes in the form of hypopigmentation, even after they were cured.

A statistical analysis was also performed on the data of the patients who had not returned the questionnaire. The non-responders were significantly younger (P < 0.0001), and interestingly a lower number of non-responders had taken a penile biopsy. Nevertheless, there was no statistical difference regarding the rate of undergoing circumcision.

**Methodological considerations**

The main drawback of this study is its retrospective nature. This could lead to selection bias and ultimately information bias. More specifically, the LS clinical features considered in this study were collected from the medical records of the patients. There is a risk that not all the existing clinical features have been recorded; however, this risk is low since the evaluation of the patients was performed by dermatologists. Another limitation of the study is that the questionnaire was answered by 59% of the participants, which could lead to bias development when interpreting the data. It seems that those with ongoing symptoms sent the questionnaire, whereas those with a complete recovery did not.

To conclude, this paper involved primarily the investigation of the clinical features for the diagnosis of LS, the number of cases where a skin biopsy procedure was performed for establishing a confirmatory diagnosis as well
as the correlation between LS and penile cancer.

As an invasive procedure, skin biopsy from the male genitalia can cause pain, bleeding, scarring and aesthetic problems. This procedure is also associated with diagnosis delay and increased laboratory costs. Moreover, the histopathological examination is not always diagnostic since in some cases may show only chronic inflammation and lack the characteristic features of LS. Regarding this, the potential risks of skin biopsy in the delicate male genital area led us to design and perform the research project published in the second paper where the aim was to investigate if RCM could be used as a non-invasive tool for LS diagnosis.

**PAPER II**

The results showed the potential of RCM in vivo for visualizing the characteristic histopathological features of LS compared with those of healthy penile skin, nonspecific balanoposthitis and plasma cell balanitis.

Our results revealed prominent fiber structures in half of the LS patients (8/16), representing hyaline sclerosis histologically. This is exemplified in Figure 6, showing that the fibers in the papillary dermis are thicker in LS than in healthy penile skin. Similar prominent fiber structures were seen in one patient with plasma cells balanitis corresponding to a scar in the investigated area.

![Figure 6.](image) The RCM data acquired from a patient with LS (a) and a healthy individual (b). As shown in the figure, fiber structures are thicker in the papillary dermis in the LS patient (a) than in the healthy individual (b). These prominent fiber structures represent hyaline sclerosis histopathologically, that can be visualized with RCM. [Size of images: 0.5 x 0.5 mm].
In RCM, a typical honeycomb pattern corresponding to the normal architecture of the stratum spinosum skin layer was found in almost all patients with LS, beside one LS patient who simultaneously had histopathologically confirmed PeIN. This pattern was also observed in healthy individuals and those with nonspecific balanoposthitis and plasma cell balanitis. Interestingly, in the LS patient with PeIN, the RCM investigation revealed instead an atypical honeycomb pattern and scattered, small, bright cells in the basal skin layer, probably representing cell dysplasia.

The comparison of the dermo-epidermal junction between LS and healthy penile skin revealed “edged papillae” in the latter group, representing the rims of bright basal cells around the dermal papillae. Nonetheless, this feature was totally absent in patients with LS.

One LS patient was first examined with RCM, and then one skin biopsy was obtained from the investigated area. This biopsy was then investigated with MPM ex vivo. The investigation revealed prominent fiber structures in the papillary dermis representing hyaline sclerosis and a typical honeycomb pattern signifying the normal architecture of the stratum spinosum skin layer. The fiber structures were brighter and sharper in the MPM images than in the RCM images.

The RCM facilitated the visualization of prominent, thick fiber-like structures, corresponding to hyaline sclerosis histopathologically, which is considered a key feature in LS diagnostics. The observation of this sclerosis feature in RCM is in line with other reports on genital and extragenital LS investigated with RCM [65, 66].

Furthermore, 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 totally absent 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 [67] and it could also explain the melanin loss in LS.

The investigation of one of the LS patients revealed an atypical honeycomb pattern in the epidermis and scattered bright cells in the basal layer. These features are commonly found in SCC [65, 68]. The histopathological
analysis of a skin biopsy obtained from the same area confirmed the diagnosis of PeIN. LS patients are followed up regularly for the signs of penile malignancy. This study supports the use of in vivo RCM as a noninvasive monitoring tool for LS patients at the risk of penile cancer as well as for the diagnosis of genital dysplasia.

**Methodological considerations**

The main limitation of this study is that sclerosis, which is the main characteristic of LS, was not identified in all LS patients examined with RCM. Several reasons account for this drawback. In the cases where RCM failed to detect sclerosis, only mild sclerosis was seen histopathologically, thus making it difficult to observe it using RCM. However, the RCM was able to identify one case of mild sclerosis. In two other cases, hyperkeratosis in the epidermis hindered the visualization of the upper dermis owing to the limitation of RCM to visualize a skin depth of 150–200 μm. Moreover, in a few cases, the RCM investigation was performed several months or years after the implementation of the skin biopsy procedure. In the elapsed time, the patients had received local treatment with a potent steroid cream, which could have altered the typical histopathological features of LS and diminished the sclerosis, thereby making it more difficult to observe. In these cases, the histological results are not directly comparable to the RCM findings.

In this study, we, for the first time, reported one case of LS investigated by MPM ex vivo. A comparison of the LS features detected with an in vivo MPM device to those detected with RCM in vivo would be more optimal. Nevertheless, since we did not have access to an in vivo MPM device, we aimed to investigate the potential of the MPM ex vivo. Consistent with the skin biopsy from a LS patient, MPM imaging revealed bright collagen fibers referring to sclerosis with greater contrast than the corresponding RCM image. Based on this result, MPM could be a better alternative for imaging sclerosis in the dermis. Future studies are warranted when an in vivo MPM is available in our department in order to confirm this preliminary result and to fully explore the potential of in vivo MPM for the diagnosis of LS.

Since the investigation was time consuming, it was not possible to examine all of the affected areas. In addition, given that the evaluation of the obtained images was also time consuming, the implementation of this device as a diagnostic tool in everyday clinical practice cannot be yet justified. Moreover, because the RCM images present a horizontal view of the skin
layers, it is difficult to directly correlate them with the histopathological images showing a vertical view of the skin.

In summary, our study showed that RCM could visualize the thick fiber structures corresponding to sclerosis in the dermis in the absence of hyperkeratosis, 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 the healthy skin and their absence in the LS patients. Importantly, RCM revealed a precursor of penile cancer in one LS patient.

In conclusion, RCM is a promising tool for diagnosing LS. It can help discriminate LS from nonspecific balanoposthitis and plasma cell balanitis if sclerosis is present. Moreover, RCM can be a valuable tool for monitoring LS patients for dysplasia, which can lead to penile cancer, reducing the number of follow-up biopsies and eliminate their potential complications.

**PAPER III**

In this study, we aimed to explore the possibilities of ex vivo MPM as a diagnostic tool for BCC.

The original aim of this project was to investigate if adding MAL, which induces endogenous formation of PpIX, could improve the imaging contrast and visualize more characteristic diagnostic features in BCCs [69]. Two-photon excitation at 780–800 nm was explored in our study, according to the settings previously reported to visualize PpIX [70]. However, this technique failed to visualize PpIX and no additional contrast was produced in BCCs. In this regard, the latter was overshadowed by the autofluorescent background. This led to a detailed investigation of the excitation and emission of PpIX.

The first experiment involved the excitation of a thin tissue section of a skin biopsy specimen obtained from a superficial BCC, previously exposed to MAL, using an ex vivo MPM at three different excitation wavelengths (i.e., 405, 710, and 810 nm). After the implementation of one-photon excitation at 405 nm, the characteristic porphyrin peak of PpIX in the emission spectrum was observed when the latter was separated from the autofluorescence spectrum. On the contrary, the use of an 810 nm excitation
wavelength did not reveal any porphyrin peak. Furthermore, the reduction of the excitation wavelength to 710 nm, led to a significant increase in PpIX and the generation of the photoproduct of PpIX emission.

Figure 7 shows a macroscopic image of a BCC, its corresponding macroscopic, fluorescence image, exposed to MAL and the image of the patient’s skin after the surgical removal of the BCC. The second experiment was focused on the ex vivo visualization of the skin biopsies obtained from surgically removed superficial BCC and healthy perilesional skin previously exposed to MAL. The obtained images with MPM ex vivo were compared to those taken from superficial BCC and healthy perilesional skin previously exposed to placebo cream.

The results were well in line with those obtained from the imaging of the thin tissue section sample in the first experiment. In this regard, autofluorescence was well-visualized using a 780-nm excitation wavelength, whereas PpIX was not visualized. On the other hand, at an excitation of 710 nm, PpIX was more apparent in the MAL-exposed samples than the surrounding healthy skin and the placebo-exposed samples. This is illustrated in Figure 8, which represents a three-dimensional reconstruction of MPM z-stacks obtained from BCCs and the corresponding normal skin.
Our third experiment was the spectral investigation of a solution of PpiX. Interestingly, the results demonstrated that the 710-nm excitation gives rise to one-photon anti-Stokes fluorescence.

**Methodological considerations**

Our results revealed a mixture of non-linear and linear dependence between the intensity and laser power, meaning that there was no pure two-photon excitation of PpIX. On the contrary, the one-photon excitation of PpIX was confirmed. Moreover, our results showed that anti-Stokes fluorescence is the dominant phenomenon when PpIX is excited. One-photon anti-Stokes excitation is a linear process resulting in the loss of excitation confinement and the elevation of the out-of-focus signal. This leads to poor resolution and loss of imaging contrast. We used a pinhole to overcome this problem; however, it did not improve the resolution. Nevertheless, the combination of the two-photon excitation of autofluorescence with the one-photon anti-Stokes fluorescence of PpIX facilitated the visualization of cellular morphology.

In conclusion, we found that wavelength of 710 nm should be used in order to excite PpIX and overcome the autofluorescent background. Adding MAL in BCCs cannot increase the contrast when imaged with MPM ex vivo. Nevertheless, our study suggests a new method that can be used in order to visualize PpIX in skin tumors.
PAPER IV

There is a need for a novel technology that can improve the work-flow and facilitate early diagnosis and tissue analysis in relation to SLN surgery. Regarding this, we aimed to explore the potential of RCM ex vivo and MPM ex vivo to identify the characteristic metastatic MM features in the lymph node tissues, given the scarcity of the studies addressing this domain \[^{71,72}\]. To this end, this exploratory study was conducted on the tissue samples obtained from a biobank, belonging to MM patients undergoing SLN diagnostic procedures at our local hospital.

The main feature identified with MPM found in all cases of MM metastasized lymph nodes was the atypical cells corresponding to the malignant cells. This feature is visualized in Figure 9.

Figure 9 illustrates MPM data obtained from a lymph node with MM metastases. Arrows shows atypical cells corresponding to malignant cells. (Photo: Despoina Kantere)
Another major finding was the nuclei pleomorphism, that is, nuclei of various shapes and sizes were consistently found in all the malignant lymph nodes. Moreover, the vessels, intravascular blood cells, and fibrous tissue of the capsule were easily distinguishable. The morphologic features observed in the lymph nodes using MPM corresponded well to the histopathologic features of MM metastasis. In addition, the morphological differences were distinct between healthy and metastatic lymph nodes. Bright spots in the nucleus, probably representing nucleus’ nucleoli were also evident in three tissue samples, including nodes positive and negative for MM metastasis.

In order to confirm the effect on observed tissue morphology based on presence or absence of paraffin, an additional comparison was performed including both metastasis positive and negative samples, as presented in Figure 10. As shown by the figure, the borders of the cells and their nuclei are better discerned in the deparaffinized tissues compared with the paraffinized ones. Moreover, bright spots in the nuclei are seen in deparaffinized lymph nodes, probably corresponding to nucleus’ nucleoli. Thus, the quality of the imaging was significantly increased when the paraffin was removed from the lymph nodes. Taken together, this analysis reveals that metastases features are more clearly visualized with MPM when paraffin is removed.

Figure 10. MPM images of lymph node with MM metastases (a, c), and from metastasis negative normal lymph node tissue (b, d). Top panel (a,b) represents images acquired with paraffin present, and lower panel (c,d) after deparaffinisation procedure.
An overview of the observed features in the lymph nodes with MPM and the comparison of these features with the histopathological counterparts is presented in Table 1.

Table 1: Overview of the observed features with MPM and a comparison of these features with the histopathological counterparts in the lymph node (LN) tissue samples. *

<table>
<thead>
<tr>
<th>Histopathological features</th>
<th>LN1</th>
<th>LN2</th>
<th>LN3</th>
<th>LN4</th>
<th>LN5</th>
<th>LN6</th>
<th>LN7</th>
<th>LN8</th>
<th>LN9</th>
<th>LN10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Parenchymal malignant cells</td>
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<tr>
<td>Parenchymal atypical cells</td>
<td></td>
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<td></td>
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<tr>
<td>Subcapsular malignant cells</td>
<td></td>
<td></td>
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<td>Subcapsular atypical cells</td>
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<tr>
<td>Nuclei polymorphism</td>
<td>Atypical nuclei</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Mitoses</td>
<td>Mitoses</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Prominent nucleoli</td>
<td>Bright nucleoli</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Homogenous cells, typical nuclei ~6 μm</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Small cells, without visible nuclei ~5 μm</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collagen</td>
<td>Brightly fluorescent fiber network</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
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</table>

* Indices (P) and (D) correspond to the imaging of paraffinized or de-paraffinized tissues respectively. Sample LN8 was excluded from the analysis due to sample processing causing artefacts.

The RCM ex vivo investigation, primarily enabled the visualization of the fiber network of the lymph nodes, however the details corresponding to cell structures could not be discerned. The results were similar for all four investigated samples, both for the paraffinized and deparaffinized ones.

Methodological considerations

Although the tissues were investigated in an unstained condition, they all have been subjected to fixation and paraffin embedding, which might alter tissue autofluorescence and signal acquisition. It was generally observed that the tissue retained in paraffin blocks exhibited lower contrast, compared to
the tissue subjected to the deparaffinization procedure before imaging. The removal of paraffin from the lymph node led to the improvement of the quality of the images and distinctiveness of the cell borders and nuclei. It was found that paraffin itself did not interfere with the autofluorescence signal. Regarding this, the reduced contrast for paraffinized tissue could be attributed to tissue dehydration, which reduces autofluorescence.

The results obtained from RCM were discouraging. This can potentially be attributed to the underlying physical principles behind the technique. The RCM is based on the backscattering of light; therefore, it is dependent on relative variations in the refractive indices and sizes of organelles and microstructures. Strong signal and bright contrast are obtained particularly from melanin, keratin, and collagen. This is suitable in the context of the skin and a differentiated epidermal structure, while for the case of the lymph node tissue, the optical properties are expected to be more homogenous. Since melanin and keratin are absent in healthy lymph node tissues and not always present in the lymph nodes with metastases, the poor performance of RCM in this type of tissue is most likely attributed due to the lack of these tissue constituents.

In conclusion, this feasibility study is the first systematic report on the potential of the label-free laser scanning microscopy for imaging MM metastases in lymph nodes. Our results show that MPM ex vivo can visualize lymph node metastases, whereas RCM ex vivo is not able to achieve that. The results of this paper show that in order to provide contrast, fluorescence-based techniques seem preferable for lymph node diagnostics. Accordingly, it can be concluded that MPM has the potential to be used as an intra-vital histological examination for this purpose.

PAPER V
The results obtained in paper IV clearly highlighted the need for improving contrast in MPM images. To accomplish this goal, in our last work, we aimed to investigate FLIM ex vivo, using both metastatic and healthy lymph nodes obtained from the same biobank utilized in the previous project.

This exploratory study reports on the data acquired by means of MPM-FLIM ex vivo. The research was conducted on five lymph node tissue samples, three of which were positive for MM metastases, and the two others were negative.
Excitation was performed at a wavelength of 780 nm. The emission was collected in red (Ch1, 550–655 nm) and green (Ch2, 500–550 nm) channels, corresponding to the autofluorescence wavelength region expected for endogenous tissue chromophores, (e.g., primarily NADH and FAD) [74-76].

The results in the MM positive lymph nodes showed pleomorphism (i.e., varying cell sizes), similar to the H&E image in all metastatic positive samples. This is illustrated in Figure 11, which shows the MPM intensity and the FLIM images obtained from one lymph node positive for MM metastases. In addition, the figure includes the values of fluorescence lifetimes acquired from the samples and the corresponding image of an H&E stained tissue section from the same sample.

Figure 11. H&E stained histologic section (a) together with MPM intensity image (b), FLIM images obtained from channel 1, 580/150 nm (c), channel 2, 525/50 nm (d) of LN positive for melanoma metastasis and corresponding fluorescence lifetime distribution histogram (f) obtained from two spectral channels. The zoomed FLIM image (e) corresponding to the region of interest (highlighted red box) in (c). Atypical cells possible undergoing mitosis (*) and randomly distributed cells (#) with short fluorescence lifetime (~600 ps) are highlighted in (e). The MPM and FLIM data were acquired at 780 nm excitation. [Field of view: (b, c, d) ~ 250x250 μm, and (e) 70 x 70 μm]. False color scale lifetime data, 256-time channels, ranging from 100–to 3000 ps. The images are used with permission from [77].

Overall, the values of the fluorescence lifetimes acquired from the samples were distributed around 100-3000 ps, and they most likely corresponded to signals from NADH and FAD, difficult to be separated due to spectral cross-talk [75]. The long fluorescence lifetime distribution of > 2000 ps most likely corresponded to FAD, while the lifetimes within the range of 300–2000 ps were probably originated from NADH [74,76]. In addition, the cells lacking visible nuclei exhibiting significantly shorter lifetime values (~ 600-700 ps) were also discerned. These cells most likely corresponded to erythrocytes. This is supported by the fact that the fluorescence lifetime distribution from the blood vessel was similar to the fast fluorescence decay of red blood cells as reported in the literature.

In the negative control lymph nodes, a more homogenous image was
visible, with predominantly hardly discernable small cells (~7 μm), most likely corresponding to lymphocytes. Interestingly, the distinct bimodal fluorescence lifetime distribution which was noticed most likely corresponds to NADH and FAD in the two different spectral channels. In this regard, the short lifetime corresponds to the free NADH and the long lifetime probably conforms to both bound NADH and FAD [75, 76]. Nevertheless, the bimodal distribution was not present to the same extent in the malignant tissue. Furthermore, the distribution between shorter and longer lifetime components was different in the two spectral channels. Thus, it has been shown that the comparison of the lifetime distributions between the two different channels can improve the diagnosis by potentially adding spectral information to the morphological interpretation.

Methodological considerations

Our data are in line with earlier reports indicating an increase in lifetimes in fixed tissues. In this study, the samples, previously subjected to fixation, were deparaffinized before the imaging but had previously undergone fixation. Since the longer lifetime was more prominent in the green channel, the signal most likely came from FAD, which is reported to have a lifetime of ~3000 ps [78]. These shifts in lifetimes for free NADH and FAD can be attributed to the fact that the investigated tissue had undergone a substantial work-up preceding the fixation and deparaffinization process [79, 80]. The same reason could account for the longer fluorescence lifetime coming from erythrocytes and blood vessels observed in our study in comparison with other reports. Another factor that may have contributed to this discrepancy is the metastatic microenvironment of the lymph node. These discrepancies observed in lifetimes mark the necessity of confirming these results in fresh lymph node tissues in order to use the MPM-FLIM technique for translation into an intra-vital tool for MM metastasis diagnostics.

In conclusion, in the last paper V, we were able to observe some features such as pleomorphism, large atypical cells, and mitosis that indicate metastasis. These features were absent in the images obtained from the healthy lymph nodes. Therefore, similar to MPM ex vivo in paper IV, MPM-FLIM ex vivo was also able to discern differences between metastasized and non-metastasized SLN by adding contrast alongside the fluorescence lifetime information. These findings showed that this technique could be a promising tool for diagnosing metastasis in an intra-vital context.
CHAPTER 9
Conclusion and Outlook for the future

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
Despoina Kantere
Conclusion and Outlook for the future

There is a need for finding reliable, accurate, fast and highly sensitive imaging techniques to facilitate the diagnosis of different dermatological and oncological disorders.

Based on the findings of this thesis the RCM in vivo and MPM ex vivo could be considered promising tools to diagnose LS and differentiate it from other genital disorders. Moreover, our results showed that adding MAL in BCCs cannot increase the contrast when imaged with MPM ex vivo. Nevertheless, we presented a new method that can be used in order to visualize PpIX in skin tumors on the verge of one-photon anti-Stokes fluorescence, by enhancing the contrast between PpIX and tissue autofluorescence. This finding is not only of importance for diagnostics, but also for future work in which photodynamic effects might be required. Finally, we, for the first time, described the morphological features of healthy and metastasized lymph nodes by means of ex vivo MPM and FLIM-MPM, showing the potential of these techniques to be used as the future intra-vital diagnostic methods in the SLN surgery.

However, our research entails several limitations that must be addressed in the future to improve the potential of the aforementioned techniques.

We used an ex vivo MPM in our research. In future studies, when accessing an in vivo MPM device, we could investigate the potential of this tool to detect and map cell dysplasia, as well as differentiating it from balanoposthitis in patients with suspected PeIN. This medium would reduce the need for obtaining multiple biopsies form the penis area, thereby accelerating the treatment process by directly referring the patient to urologists for undergoing surgery. Furthermore, we could explore the potential of this technique on investigating lymph nodes during the SLN surgery.

Another problem that has to be addressed in the future is the limited
imaging depth of both RCM and MPM which is approximately 200 μm. Due to this limitation, there is no feasible way, at this stage, to image the whole dermis of the skin and the whole lymph node. This limitation cannot be overcome by increasing the excitation intensity. Currently, biocompatible fluorescent probes and novel compact laser sources for optical windows ranging from 1000 to 1870 nm are under development. These technological advances would allow deeper tissue imaging than the current Ti:sapphire lasers, with the optical window of 600–1000 nm \cite{81}.

Future research should also be focused on improving contrast, sensitivity, and specificity in identifying malignant cells in the lymph nodes. In our last study, we showed that by combining MPM with FLIM ex vivo we can facilitate the achievement of an additional contrast to discern morphological features such as pleomorphism, lymphocytes, blood vessels, and erythrocytes. The next stage would be to proceed to perform experiments with MPM and MPM-FLIM ex vivo using fresh lymph nodes removed from mice, and at a later stage, investigating human lymph nodes in order to validate our data. Combining MPM with other optical label-free imaging techniques such as CARS (coherent anti-Stokes Raman scattering) can also provide more details on the tissue structure \cite{82}.

Another strategy to achieve more contrast in imaging, would be to combine nanotechnology with biomarkers. Specific melanoma markers, such as S-100, MART-1, HMB45, and MCAM/MUC18, could be conjugated with gold nanoparticles (AuNPs) to accomplish this goal.

At last but not least, another issue to be addressed in future research is the use of big data produced by the laser scanning microscopy of biological tissues. It is an extremely time-consuming task for the researchers to evaluate a large number of produced images, especially when evaluating inflammatory skin disorders that are widely spread. Artificial intelligence could be the answer to this big challenge. The recently developed convolutional neural networks are performing on par with humans in image recognition and segmentation applications \cite{83, 84}. More specifically, the difficulty in reading RCM and MPM images is a barrier to training and wider adoption of this technology in every clinical practice. To address the need for training, machine learning-based image analysis is being investigated to provide a more quantitative and objective approach for image analysis. There are already reports on the development of new methods for the automated identification of diagnostically
significant areas in the RCM images of melanocytic lesions \cite{85}. In addition, some research has been focused on developing new automated algorithms that quantitatively guide the delineation of the dermo-epidermal junction \cite{86}. Further development of such algorithms is needed to enable the identification of both normal and abnormal morphological features in human tissues.

In conclusion, we are heading towards a new era in medical diagnostics, where laser scanning microscopy techniques can help intercept diseases by diagnosing them within a shorter period of time and in a non-invasive manner.
Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy
Epilogue

Are you reading the epilogue now? Congratulations. If you got here, then you realize one thing, what I finally realized. That is, the research stories are just like the fantasy stories, they have a beginning, a plot with surprises and twists, and an end. It is just a little different because to my great surprise and emotion, I finally realized that these research stories never end, they just keep going from articles to articles and books to books, and so on, for all the years that have passed and all the years that are yet to come.
CHAPTER 11
Acknowledgments

Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy

AUTHOR
Despoina Kantere
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Diagnostic aspects of lichen sclerosus and skin cancer studied with laser scanning microscopy
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