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In vivo Reflectance Confocal Microscopy

Innovations in Skin imaging

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In vivo Reflectance Confocal Microscopy: Innovations in skin imaging

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General introduction, aims and outline of this thesis
Skin

The skin is the largest organ of the human body, it has a surface area of 1.5-2.0 m² and accounts for 15% of the total body weight. It serves as the interface between the human body and its environment. One of the main functions is to protect against pathogens and ultraviolet light (UV). Skin regulates body temperature, contains sensory receptors and has a role in the immune system. These functions are achieved through the unique multilayered composition of the skin, a cellular epidermis and underlying dermis and hypodermis (Figure 1). In the epidermis, different layers can be distinguished. From the inside to the outside: stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (SC). This multilayered epidermis mainly consists of keratinocytes, which develop in the basal layer of the epidermis and differentiate throughout the epidermis while migrating to the outermost layer. In this layer keratinocytes have lost their nuclei. These tightly packed flattened anucleated cells full of keratin (corneocytes) form the SC, which mainly regulates the physical protection of the skin. To protect against UV light, melanocytes are present in the epidermis. The dermis is made of connective tissue and contains few inflammatory cells to regulate cutaneous inflammatory responses. The hypodermis, or subcutis, consists of loose connective fatty tissue. It attaches the skin to the sub-adjacent tissue, plays an important role in thermoregulation and also functions as shock absorber.

Pathology of the skin

Skin cancer

Skin cancer is the most common cancer and its incidence is increasing rapidly in Western countries. In 2008, it was predicted that in the Netherlands, 1 out of 5-6 individuals will develop skin cancer before the age of 85.
Chapter 1: General Introduction and Outline of This Thesis

The majority of malignant skin tumors can be divided in melanoma and non-melanoma skin cancer (NMSC). Melanoma skin cancer represents 10% of all skin cancer and accounts for at least 65% of all skin cancer-related deaths. NMSC represents the other 90% and comprises mainly basal cell carcinomas (BCC), squamous cell carcinomas (SCC) and its precursors, actinic keratosis (AK) and Bowen disease (BD) (Figure 2). SCC account for 25-35% of the yearly skin cancer-related mortality. BCC in contrast, have very low metastatic potential associated with low mortality. However, NMSC may cause high morbidity and extensive disfigurement. The incidence ratio between BCC and SCC is 4:1. Nowadays, the number of SCC and melanoma per year are estimated at approximately 19,000 and 5,443, respectively. With respect to the pre-malignancy AK, it is estimated that 1:4 of the 16 million Dutch citizens are affected. It is predicted that incidences of skin cancer will rise with 6-8% per year, depending on the tumor type. As a result of this, it is evident that skin cancer places an enormous burden on the healthcare system with increasing costs.

Genotypic, phenotypic and environmental factors increase the risk on developing skin cancer. However, the major risk factor is exposure to UV. UV radiation causes direct damage to DNA and RNA. Therefore, sun protection, especially during childhood, is important. In case of clinical suspicion of a BCC or SCC, the skin lesion will be biopsied or excised for pathological confirmation of the diagnosis. Currently, the evaluation of a biopsy specimen by a pathologist is the gold standard in diagnosing NMSC. Unfortunately, this procedure is invasive and it will require up till one week before the diagnosis is made. The treatment is determined based on the diagnostic biopsy and depends on the tumor (sub)type, size, depth and location and patient related factors like age. As pathologists investigate only a few slides of the biopsy specimen, and a biopsy only represents a small part of the tumor, this might result in sampling errors, leading to inappropriate chosen treatments or margins when excising the tumor. Moreover, as nowadays several new non-surgical treatment modalities (photodynamic therapy, Imiquimod, 5-fluorouracil cream etc.) are used, it is very important that the diagnosis is correct, because the pathological confirmation of an excised tumor is missing in these situations.

The continuing increase of skin tumors will place a high burden on our health care system, leading to longer waiting lists, resulting in treatment delay. Cost-effective and efficient skin cancer care requires extensive prevention methods, appropriate treatments and importantly, accurate and cost-effective diagnostics. In this thesis, studies have focussed on non-invasive diagnosis of NMSC.

Dynamics in skin inflammation

Several diseases of the skin are known which represent inflammatory processes. For an overview of various inflammatory skin diseases the reader is referred to the textbooks of Dermatology. Since many cell types and mechanisms are involved, it is challenging to unravel the pathogenesis of these inflammatory diseases. In this thesis, studies have

Figure 2. Skin cancer lesions (1) and corresponding hematoxylin-eosin stained tissue section (2). a) Nodular basal cell carcinoma. b) Superficial basal cell carcinoma. c) Actinic keratosis. d) Squamous cell carcinoma.
focussed on the dynamics of neutrophils in psoriasis, which is a disorder with hyperactivity of innate immunity and signs of activation of acquired immunity.\textsuperscript{19}

The presence of several types of immune cells has been demonstrated in psoriatic lesions, providing scientific evidence supporting the theory of a dysregulated immune system in this skin disease.\textsuperscript{20–24} Currently, psoriasis is predominantly considered to be a disorder of innate immunity. The recruitment and activation of preferentially \( T \) helper-1 cells by natural killer \( T \)-cells, dendritic cells and keratinocytes play a key role in the pathogenesis of psoriasis.\textsuperscript{25–27} Further accumulation of polymorphonuclear neutrophils (PMN) in the living epidermis (spongiform pustule of Kogoj) and in the corneal layer (microabscesses of Munro) are characteristic features in psoriasis and are thought to play a role in the pathophysiology of this skin disease. This accumulation of neutrophils is a dynamic process and occurs in several consecutive phases.\textsuperscript{23–25} It is known that growth related oncogene \( \alpha \) and interleukin-8 are major chemo-attractants that contribute to neutrophil diapedesis and migration.\textsuperscript{25–28} Production of interleukin-8 by keratinocytes is enhanced by interleukin-17, which is made in large amounts by PMN. Interleukin-17 has a major role in the pathogenesis of psoriasis.\textsuperscript{27, 28} Drugs that decrease neutrophil counts and migration are highly effective in the treatment of psoriasis.\textsuperscript{29–32} Although neutrophils are thought to play an important role in the pathogenesis of psoriasis, signals for neutrophil diapedesis and migration in vivo are not yet fully understood. To unravel this dynamic in vivo process, serial biopsies may exhibit some information. However, obtaining invasive biopsies will be regarded as unethical. Furthermore, they only provide static (immuno)-histological data, which will be influenced by the inflammatory processes in the skin caused by the biopsy procedures. Therefore, non-invasive in vivo models and techniques could be of value in clarifying dynamic inflammatory processes.

**Skin damage**

Mechanical skin damage induced by skin-material interaction, disrupting the skin barrier function, is hardly studied. The disruption of the skin barrier function is often only studied at biological level in field of skin disorders or burn wounds.\textsuperscript{31, 34} However mechanical skin damage is regularly occurring. A sliding during sports can e.g. cause this kind of mechanical skin damage, but is rarely described in literature. Also pressure ulcers are caused by interaction (pressure and shear) of skin with a material.\textsuperscript{35} However, earlier studies on skin-material interaction focussed on the material that caused the skin damage, whereas clinical and morphological changes with respect to skin damage were not examined. A more complex but probably more accurate way to study skin damage would be to use the skin itself as a readout system. Such a model, which should be non-invasive, will result in more knowledge and understanding about growth, differentiation, inflammation and mechanical disruption in skin damage. In this thesis, studies have therefore focussed on obtaining morphological data on skin damage and on development and validation of an in vivo method to study skin damage.

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**In vivo skin models**

In order to study one specific process or cell type of the skin, skin models are used to simplify a complex in vivo situation. In vitro skin models can be investigated continuously and provide a quite dynamic picture, but lack the complex in vivo environment. Therefore, non-invasive or minimal invasive in vivo models in which a specific response can be evaluated are often used. Studies in this thesis, have focussed on the tape stripping and leukotriene B4 (LTB4) model.

**Tape stripping**

Tape stripping is a commonly used in vivo model for studying skin barrier function, epidermal growth and concurrent immune responses.\textsuperscript{36, 37} This model is a minimal invasive procedure in which the SC is removed and results in skin barrier disruption. It consists of sequential application and removal of an adhesive tape onto the skin surface until the skin glistens. In this way, microscopic layers (0.2-1µm) of the SC are removed and provides standardized trauma of the skin.\textsuperscript{38–40}

**Leukotriene B4**

Epicutaneous application of human LTB4 is an established in vivo model that locally induces skin inflammation. Leukotrienes are intracellular signaling molecules that are overproduced during an allergic and inflammatory response in several tissues, including the skin.\textsuperscript{41–43} Leukotrienes are primary made by inflammatory cells like neutrophils, basophils, eosinophils, monocytes, macrophages, and mast cells.\textsuperscript{41, 42} They are metabolites of arachidonic acid derived from the 5-lipoxygenase pathway.\textsuperscript{44–46} LTB4 has been shown to have potent chemo-attractant activity for PMN, resulting in PMN infiltration into the skin 24 h after topical application, which subsequently is followed by a mono- nuclear infiltrate in the dermis between 48-72 h.\textsuperscript{45, 47–49} Topical application of LTB4 causes a dose dependent acute response and attracts a homogenous population of inflammatory cells. Therefore, this model is useful in studying the specific role of PMN in inflammatory skin diseases like psoriasis.\textsuperscript{47–49}

Currently in both the tape stripping as well as the LTB4 model, a skin biopsy is required to evaluate the morphological and (immuno)-histological changes in the skin. Even more invasive, to study processes over time, several biopsies are needed. Besides the invasiveness, the same location cannot be followed and most importantly the biopsies induce inflammation by itself, which interfere with the study results.
Non-invasive dermatological diagnostics

As described above, invasive biopsies have certain disadvantages in both research and the clinic. Therefore, several non-invasive dermatological diagnostic tools have been developed and investigated. Besides dermoscopy, Raman spectroscopy is one of these techniques. Raman signals correlate with the molecular vibrations of various tissue biomolecules. This technique seems to be capable to detect molecular and/or biochemical changes associated with pathology. Secondly, fluorescence diagnosis with aminolevulinic acid-induced porphyrins (FDAP) offers the opportunity to study various tissue types in vivo. This technique is based on differences in the ability to accumulate the endogenous photosensitizer protoporphyrin-IX (PpIX) after incubation of the skin with alpha lipoic acid. As PpIX has characteristic fluorescence properties, its preferential accumulation in certain tissue types can be used as diagnostic tool. Although FDAP seemed promising, in skin it is difficult to discriminate between keratinocytic intraepidermal neoplastic lesions or inflammatory and proliferative activity. Optical coherence tomography is another non-invasive imaging technique, which is based on interferometry. The principle is comparable to ultrasound, but instead of longitudinal ultrasound waves, infrared-light is used, yielding an axial and lateral resolution of approximately 15 µm and a penetration depth of 500-1000 µm. Although inflammatory dermatoses and skin cancer can be visualized, no cellular or subcellular details may be seen, only architectural changes of the skin can be visualized. The basement membrane cannot be distinguished, such that early tumor invasion cannot reliably be determined. In vivo reflectance confocal microscopy (RCM), a non-invasive technique for imaging skin, however, offers a resolution corresponding to conventional light microscopy.

History of reflectance confocal microscopy

The confocal microscope was invented by Marvin Minsky in 1957. At that time, the microscope was used to image ex vivo samples. Over time the device was adapted by using a white light source and a spinning disk of pinholes in order to image human skin in vivo. Other improvements were the use of a laser light and a spinning polygon mirror. The technique with the spinning polygon mirror has been commercialized by Lucid Inc. (Rochester, New York, USA) in 1997. Their devices, the VivaScope 1500 and 3000, are currently the most often used RCM devices with respect to dermatological patient care and research (Figure 3).

Principle of reflectance confocal microscopy

The reflectance confocal microscope uses a point light source, derived from a near-infrared laser of 830 nm, which illuminates a horizontal plane within the tissue. The laser power on the skin lies between 5 and 10 mW and does not cause any tissue damage. A detector will catch the reflected light from the tissue through a small aperture (pinhole). Out of focus light will be blocked from detection by this pinhole. The point source of the light, the illuminated spot in the tissue, and the pinhole aperture are situated in optically conjugate focal planes (Figure 4). The measured lateral resolution of RCM has shown to be 0.5-1 µm and the axial resolution less than 5 µm. This technique produces horizontal (en face) images of the skin and can examine the skin till a depth of 250 µm.

Figure 3 Vivascope 1500. A metal ring with an adhesive window is applied to the skin. The Vivacam will be used to obtain a dermatoscopic image. Ultrasound gel will be applied on the window and the objective lens housing will be attached to the magnetic metal ring. After starting the laser, black and white images will appear on the computer screen of the device.
white blood cells, chromatin, collagen, elastin is lower (Figure 5). Layers of normal skin can be distinguished clearly (Figure 5), various skin types will however result in minor reflection differences.\textsuperscript{62-64} Layers of normal skin can be distinguished clearly (Figure 5), various skin types will however result in minor reflection differences.\textsuperscript{62, 65}

Reflectance confocal microscopy in dermatology
Melanin and melanosomes are strong sources of contrast for RCM images. These compounds and cell organelles are key features of nevi, melanomas, pigmented BCCs and solar lentigines.\textsuperscript{15, 66-69} For this reason, RCM is mainly used to diagnose melanocytic lesions at this moment.

Besides diagnosing melanocytic lesions, RCM features for several non-melanocytic lesions are described. In AK, inhomogeneous, irregular SC, irregular honeycomb pattern of keratinocytes, loss of regular stratification of the epidermal layers, and dyskeratotic areas can be seen.\textsuperscript{66-69} At the dermal level, RCM images of AK show thick bundles consistent with solar elastosis.\textsuperscript{72} The confocal features of SCC include polygonal nucleated cells at the stratum corneum, atypical honeycomb or a disarranged epidermal pattern, as well as modification of vascular patterns.\textsuperscript{72, 73} Until now, RCM does not allow discrimination between hyperkeratotic AK and SCC or between superficially invasive SCC and SCC in situ ( Bowen’s disease). Described RCM features for BCC include islands of monomorphic elongated basaloid cells with nuclei that are orientated along the same axis.\textsuperscript{63, 72, 74, 75} These tumor nests are often surrounded by cleft-like dark spaces, bright stromal tissue and the
presence of multiple inflammatory cells. Further, elongated monomorphic keratinocyte nuclei can be found that are polarized along one axis. Additionally, prominent enlarged capillaries with a horizontal orientation can be observed surrounding the tumor nests. Leukocyte rolling can be visualized within the capillaries.

In experienced hands, diagnosis by RCM will require 5-10 minutes and can dramatically reduce (68%) unnecessary biopsies and excisions of benign lesions. In addition, RCM can be useful in distinguishing between nodular appearing lesions, which can be clinically difficult. Besides diagnosis of all kind of skin lesions, it is shown that RCM is a useful tool for monitoring and follow-up of therapy in skin cancer as well as inflammatory skin diseases.

In dermatology, RCM offers several advantages over conventional histology. Imaging is painless and non-invasive, causing no tissue damage. The skin is not altered by tissue processing (fixation, sectioning, and mounting) or staining, which may cause disruption of the native structure of the skin, thus reducing interpretive ambiguities. Moreover, an inflammatory response, which may interfere with the diagnosis and study observations will be prevented. Further, real-time data collection is faster than routing histology and the same location of the skin can be repeatedly imaged over time. This allows to evaluate dynamic changes such as tissue growth, wound healing, lesion progression or response to therapy. However, the establishment of reproducible correlations between conventional histology and RCM is an absolute requirement to support implementation of RCM in the general dermatology.

**Aims and outline of this thesis**

The major objective of this thesis was to study innovative applications of non-invasive RCM imaging in the field of skin cancer and inflammation. Further, the morphology of skin damage was explored using non-invasive methods. In these studies we aimed to develop and validate non-invasive models for skin research.

Until now, the use of RCM was mainly focused on diagnosis of melanoma. In chapter 2 we aimed to explore the use of RCM in the field of NMSC, focusing on non-invasive in vivo diagnosis of BCC subtype (chapter 2.1) and distinction between benign intradermal nevi and nodular BCC. (chapter 2.2) In addition, the question was addressed whether there are specific RCM features that allow real-time differentiation between AK and SCC. (chapter 2.3) As clinical application, the use of RCM as tool to obtain a guided biopsy was tested. (chapter 2.4)

Understanding the pathogenesis of neutrophilic conditions will contribute to development of new, skin and patient friendly therapies. However, studying dynamic inflammatory processes in the skin in vivo is hardly possible as biopsies themselves will cause an inflammatory response and therefore prohibit sequential analyses. For this reason, the purpose of chapter 3 was to evaluate the use of non-invasive RCM in monitoring dynamic inflammatory processes in the skin. Psoriatic lesions were evaluated for the presence of Munro microabscesses and in repeated analyses the dynamics were studied. (chapter 3.1) Since, accumulation of inflammatory cells appear random in diseased skin a reproducible induction of neutrophil influx in the epidermis in vivo was studied. skin inflammation induced by the epicutaneous application of leukotriene B4, yielding a selective influx of PMN cells with a highly reproducible phasing. (chapter 3.2)

Information on skin damage, often occurring after skin-material interaction, is scarce. When described, only the clinical appearance has been mentioned and morphological data are lacking. In chapter 4 we aimed to fill this gap in knowledge by giving insight in the micro-morphology of the skin resulting from skin-material interactions. (chapter 4.1) Ethical considerations do not allow studies in which multiple skin biopsies are required. For obtaining morphological data on skin damage caused by skin-material interaction the aim was to develop a non-invasive in vivo model and to study the opportunities and restrictions of RCM in this model. (chapter 4.2)

In chapter 5 the general results obtained in this thesis with RCM are discussed in the light of future positioning of this innovative non-invasive approach.
References


13. Houman TS, Feldman SR, Willford PM et al. Skin cancer is among the most costly of all cancers to treat for the medicare population. Journal of the American Academy of Dermatology 2003;48;423-429.


CHAPTER 1 GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS


Non-melanoma skin cancer
2.1

*In vivo* diagnosis of basal cell carcinoma subtype by reflectance confocal microscopy
Abstract

Background: Reflectance confocal microscopy (RCM) is a non-invasive imaging technique. Currently, RCM is mainly used for the diagnosis of melanoma and non-melanoma skin cancer including basal cell carcinoma (BCC). Until now, it has not been possible to distinguish between subtypes of BCC using RCM.

Objective: To establish the RCM features for subtypes of BCC.

Methods: 57 lesions were selected for RCM imaging. Clinical and dermatoscopic pictures were taken and a 3-mm biopsy was obtained.

Results: It was demonstrated that tumor nests with peripheral palisading, branch-like structures, fibrotic septa and increase in vascular diameter were characteristic RCM features for nodular and micronodular BCC. The size and shape of the tumor nests allowed further distinction between these BCCs. Solar elastosis and tumor nests connected with the basal cell layer characterize superficial BCC.

Conclusion: This study presents RCM features for BCC, which might allow in vivo diagnosis of the nodular, micronodular and superficial subtype of BCC. This could prevent a skin biopsy, resulting in direct proper treatment. Further, RCM allows to evaluate the total lesion, which makes it possible to detect mixed-type BCCs.

Introduction

Basal cell carcinoma (BCC) is the most common skin cancer.1,3 BCC accounts for 70–75% of all cases of non-melanoma skin cancer with a varying incidence over the world.1,4 This incidence is rising rapidly with 10% each year worldwide.1,5

Clinically, BCC is characterized by pink to red-brown patches or papules, central erosion or ulceration with or without the presence of crusts, a pearly shine and a raised border with the presence of telangiectasias.6 BCC can be divided into 4 main subtypes based on the histopathological growth pattern nodular (nBCC), micronodular (mnBCC), infiltrating (iBCC) and superficial (sBCC).2

At the histopathological level, nBCC shows large nests of basaloid cells in either the papillary or reticular dermis. Tumor nests of mnBCC have a similar appearance as nBCC, but they are smaller and more widely spread into the dermis. Further, these tumor nests are accompanied by prominent stromal proliferation. iBCCs appear as irregularly sized and shaped nests of tumor cells. The tumoral stroma of these BCCs is often fibrotic with plump proplastic fibroblasts.2,7 sBCC is characterized by multifocal nests of basaloid tumor cells, and it predominately shows horizontal growth and only infiltrates the upper papillary dermis.2

Determination of the subtype of BCC is important for the choice of treatment and determination of the surgical excision margin. Worldwide, surgical excision is the most common treatment option for BCC. In Europe, the margin for excision depends on the subtype of BCC. In the Netherlands, nBCC and sBCC are removed with a margin of 3-mm. In contrast, the more aggressive BCC subtypes mnBCC and iBCC are excised with a margin of 5 mm. In addition to excision, non-invasive therapies like photodynamic therapy and topical application of imiquimod are available and are increasingly applied.9,11 These therapies may be used in sBCC or nBCC, when surgery is considered suboptimal: in patients with multiple comorbidities, a high risk of postsurgical hypertrophic scarring with or without functional impairment, large lesions in functional areas or when the cosmetic outcome is of major concern.1,10 The different excision margins and the increasing use of non-invasive therapies makes correct evaluation of the BCC subtype more important. However, clinical examination is limited in its ability to distinguish between histopathological BCC subtypes.1,7 Currently, the gold standard for establishing the diagnosis of a BCC and the subtype is the evaluation of a skin biopsy by a pathologist.

Reflectance confocal microscopy (RCM) is a non-invasive technique for in vivo imaging of the skin that uses a near-infrared laser beam of 830 nm. This technique produces horizontal (en face) images of the skin.14,15 The resolution of these images is comparable with conventional histology.15 Nowadays, RCM imaging is mainly used for the diagnosis of melanoma and non-melanoma skin cancer.16,17 Besides, RCM has been shown to be a useful tool for monitoring and follow-up of therapy of inflammatory skin disorders and BCC.2,23
Until now, several RCM features of BCC have been described. RCM can visualize islands of monomorphic elongated basaloid cells with nuclei that are orientated along the same axis.1, 14, 15, 24, 25 These tumor nests are often surrounded by cleft-like dark spaces, bright stromal tissue and the presence of multiple inflammatory cells.14, 25, 26 Further in the stratum spinosum, elongated monomorphic keratinocyte nuclei that are polarized along one axis can be found.1, 23 Additionally, prominent enlarged capillaries with a horizontal orientation can be observed surrounding the tumor nests.1, 14, 15, 24, 25 Although these RCM features allow to confirm the diagnosis of BCC, it is currently not feasible to differentiate among the various histopathological subtypes of BCC.14 Therefore, the aim of this study was to evaluate whether RCM is able to distinguish between subtypes of BCC.

### Materials and methods

**Subjects**

In 34 patients, 57 clinically suspicious lesions for BCC were included in this study between December 2011 and April 2013. Patients were recruited from the Department of Dermatology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands. This study was approved by the local medical ethics committee, which was conducted according to the principles of the Declaration of Helsinki. If required, patients gave their written informed consent before inclusion. The lesions were located on the face, neck, scapula, chest, abdomen, back, arms and legs.

**Study Protocol**

Clinical and dermatoscopic pictures of the lesions were taken prior to RCM imaging using a digital camera (Nikon D 200, Nikon, Tokyo, Japan) and a Vivacam Macro Camera (Vivacam; Lucid Inc., Rochester, N.Y., USA). These photographs were evaluated by an experienced dermatologist. RCM imaging was performed according a standardized protocol. A horizontal map of 4 x 4 mm (Vivastack) was made at the level of the stratum corneum, stratum granulosum, stratum spinosum, dermal-epidermal junction and papillary dermis. Vertical mapping (Vivastack) was performed by capturing a series of images of 0.5 x 0.5 mm in depth with steps of 4.5 μm. The mapping started at the top of the stratum corneum until the papillary dermis. Movies were made at the level of the dermal-epidermal junction to visualize capillary blood flow. RCM images were evaluated for features of BCC: tumor nests surrounded by peritumoral dark spaces, peripheral palisading, elongation of nuclei along the same axis, keratinocyte atypia with spongiosis, solar elastosis, increased vascularization, presence of inflammatory cells and leukocyte rolling. In addition, the lesions were evaluated for RCM features specific for BCC subtypes. After RCM imaging, 3 mm punch biopsies of the lesions were obtained for diagnosis by a pathologist. Local anesthesia was used with 1% xylocaine/adrenaline. The histopathological subtype was used for correlation to the RCM images.

**RCM Imaging**

RCM imaging was performed with the commercially available VivaScope 1500 system (Lucid Inc.). This system produces en face images of the skin and is able to examine the skin till a depth of 200-250 μm. Images were obtained and analyzed using Vivascan 7.0 software. A more detailed description of the system has been published previously.25, 26

**Histology**

The histopathological classification of BCCs was performed according to the Dutch guideline.30 sBCCs were identified by nests of basaloid cells residing highly in the epidermis, usually in a multifocal pattern. nBCCs were characterized by large nests with basaloid cells, well circumscribed from the surroundings. mnBCCs were identified by well-circumscribed, small nests of basaloid cells, with a size comparable to the bulb of a hair follicle. The skin samples were embedded in paraffin after a 4 h fixation in formaldehyde. Paraffin sections (6 μm) were processed side by side and dewaxed with Histosafe (Adamas, Rhemen, The Netherlands) followed by rehydration in decreasing concentrations of alcohol (from 100 to 50%) and demineralized water. These sections were hematoxylin-eosin (HE) stained for assessment of histopathological features. Finally, the sections were photographed at a magnification of 20x using a microscope (Axioskop2 MOT; Zeiss), digital camera (Axiocam MRc5; Zeiss) and AxioVision software (Zeiss).

**Results**

**Diagnosis**

All biopsies of the lesions suspect of being BCC were evaluated by a pathologist. Forty-three biopsy specimens from 27 patients contained histopathological features of BCC. The clinical characteristics of the study population are summarized in Table 1. Twenty-three biopsy specimens were characterized as sBCC, 11 as nBCC and 3 as mnBCC. Six biopsy specimens showed a mixed-type BCC. Three out of 6 mixed-type BCCs contained a superficial and nodular component, the other 3 contained a nodular and infiltrative component. RCM imaging was not possible in 2 sBCC lesions, due to hyperkeratosis, and these lesions were excluded from analysis. The remaining 14 lesions were diagnosed as actinic keratosis, unspecific chronic inflammation or sebaceous gland hyperplasia. Eleven of these 14 clinically suspicious BCC lesions were not suspect of being BCC based on RCM imaging.

**RCM Features of BCC**

The images were first evaluated for general RCM features of BCC (Table 2). In 40 histopathologically confirmed BCC lesions, tumor nests were found (97.6%). Frequently, these nests were surrounded by peritumoral dark spaces (80.9%). Further, keratinocyte atypia was observed in all BCC subtypes. In 92.7% of the BCCs, increased vascularization was....
structures were rarely found in sBCC lesions (9.5%). Further, the vascular diameter was more prominent in mnBCC compared to nBCC. These branch-like structures appeared as highly reflective bands within the tumor nests (Figure 1e). The reflectivity of these structures within the tumor nests in 71.4% of all nBCC and mnBCC lesions. These branches hardly found in sBCC. In addition, RCM imaging showed highly reflective branch-like structures within the tumor nests in 71.4% of all nBCC and mnBCC lesions. These branches were found large highly reflective cells. These cells were mainly found in the upper dermis and do not correspond to any known histopathological feature. In 43.9% of all BCCs, we found large highly reflective cells. These cells were mainly found in the upper dermis surrounding or near BCC nests (Figures 1d and 2d).

Some RCM features were related to a specific subtype (Table 2). The presence of peripheral palisading, solar elastosis, branch-like structures, fibrotic septa, location, size and shape of tumor nests might be important to distinguish between nBCC, mnBCC and sBCC. Remarkably, peripheral palisading was predominately found in nBCC and mnBCC (78.6 % of all nBCCs and mnBCCs) opposed to sBCC (9.5% of all sBCC lesions). Further, we found a difference in the size and shape of sBCC, nBCC and mnBCC tumor nests. All sBCC and nBCC lesions appeared as round to oval nests (Figures 2f and 3e, Table 2). Most of these nests had a diameter of >300 μm. In contrast, mnBCCs showed tumor nests with a size similar to a bunch of grapes, varying between 100 and 300 μm (Figures 1e and f, Table 2). The size and shape of the tumor nests corresponded highly to the nests seen in histology (Figures 1c, 2c and 3c). Fibrosis was found in all subtypes of BCC. However, nBCC and mnBCC showed fibrotic septa surrounding nests of basaloid cells, which were hardly found in sBCC. In addition, RCM imaging showed highly reflective branch-like structures within the tumor nests in 71.4% of all nBCC and mnBCC lesions. These branches appeared as highly reflective bands within the tumor nests (Figure 1e). The reflectivity of these structures was more prominent in mnBCC compared to nBCC. These branch-like structures were rarely found in sBCC lesions (9.5%). Further, the vascular diameter was found and these capillaries had mainly a horizontal orientation. Other general RCM features of BCC were solar elastosis (65.9%), fibrosis (63.4%), presence of inflammatory cells (82.9%), peripheral palisading (39%) and streaming of keratinocytes (41.5%). The evaluated general RCM features of BCC correlated highly with histopathological features seen in the biopsies. However, streaming of keratinocytes is a characteristic only seen with RCM and does not correspond to any known histopathological feature. In 43.9% of all BCCs, we found large highly reflective cells. These cells were mainly found in the upper dermis surrounding or near BCC nests (Figures 1d and 2d).
In a part there was an increase in vascular diameter. The RCM diagnosis based on these findings was in line with the diagnosis based on the excision specimen.

For the mixed-type BCCs containing a nodular and infiltrative component, mainly the nodular component was visualized. Based on the histology of these mixed-type BCCs, the infiltrative component was mainly located in the deeper dermis, which is not possible to visualize by RCM. RCM imaging showed large round to oval nests of basaloid cells with peripheral palisading in the upper dermis, referring to the nodular component.

larger in nBCC and mnBCC compared to sBCC. In sBCC, the location of the nests is important. Clusters of basaloid cells were visualized just below or connected to the basal cell layer (Figure 2 f). This is in contrast to nBCC and mnBCC nests, which were found in the papillary dermis. More solar elastosis was found in sBCC compared to both nBCC and mnBCC. This solar elastosis was seen as a highly reflective network of collagen fibers (Figure 2 e).

For the mixed-type BCCs containing a superficial and nodular component, RCM features of both BCC subtypes were observed. Large round to oval nests were found just below the epidermis as well as deeper in the dermis. Further, mild to severe solar elastosis was found and in a part there was an increase in vascular diameter. The RCM diagnosis based on these findings was in line with the diagnosis based on the excision specimen.

For the mixed-type BCCs containing a nodular and infiltrative component, mainly the nodular component was visualized. Based on the histology of these mixed-type BCCs, the infiltrative component was mainly located in the deeper dermis, which is not possible to visualize by RCM. RCM imaging showed large round to oval nests of basaloid cells with peripheral palisading in the upper dermis, referring to the nodular component.
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BCC subtypes is becoming more important to select the appropriate treatment. This will result in direct accurate treatment and therefore could reduce the BCC recurrence rate. Therefore, the purpose of this study was to define confocal features characteristic of subtypes of BCC to allow a non-invasive diagnosis.

We evaluated the RCM features of 41 histopathologically confirmed BCCs. Importantly, RCM features of nBCC, mnBCC and sBCC were defined. It was demonstrated that tumor nests (aggregations of basaloid cells) with peripheral palisading, branch-like structures, fibrotic septa and increase in vascular diameter were the main characteristic RCM features for nBCC and mnBCC. The size, shape and location of the tumor nests allows further distinction between nBCC and mnBCC. Solar elastosis and the location of the tumor nests just below or in connection with the basal cell layer characterize sBCC. Although this study shows that RCM might allow in vivo diagnosis of nBCC, mnBCC and sBCC, RCM features for iBCCs still need to be defined. It was impossible to define RCM features for iBCC based on the included mixed-type BCC with an iBCC component in the deeper dermis. Unfortunately, we were not able to include single-type iBCCs. However, the incidence of so few iBCCs fits the epidemiology of BCC in general. In addition to the described RCM features for the subtypes of BCC, we found large highly reflective plump bright cells in a part of the evaluated BCCs. These plump bright cells might be inflammatory cells. However, these cells were larger and different in shape compared to the already described inflammatory cells in BCC like lymphocytes and dendritic cells. These cells might be melanophages. With RCM these cells appear as bright, plump, oval or star-shaped cells with no visible nucleus and illdefined edges. This RCM description corresponds highly to the observed plump bright cells surrounding the BCC nests but should be confirmed by immunohistochemical staining.

RCM imaging can still be time consuming, a learning curve is associated with it, and the use depends on the location of the lesion. Adequate protocols describing the most important RCM features may facilitate and shorten the procedure. Especially, RCM imaging is of additional value in diagnosing mixed-type BCC, previously treated or other clinical indistinctive lesions. In contrast to a biopsy, with RCM it is possible to scan the complete lesion non-invasively, which potentially prevents missing a more aggressive part of a tumor in BCCs located in the upper dermis.

In conclusion, this study presents RCM features for BCC, which might allow in vivo diagnosis of the nodular, micronodular and superficial subtype of BCC. This could prevent a skin biopsy, result in direct proper treatment and establishment of the correct margin for excision. Further, it was demonstrated that it is possible to detect mixed-type BCCs by RCM.

Discussion

RCM has proven to be a useful, non-invasive tool for the in vivo diagnosis of skin tumors and inflammatory skin disorders. The confocal features of melanoma and non-melanoma skin cancer have been reported previously, including general features for BCC. However, distinctive features for the subtypes of BCC have not been reported. The increasing use of non-invasive therapies, mainly in Europe, is only suited for certain subtypes of BCC. For this reason and the different excision margins, correct evaluation of BCC subtypes is becoming more important to select the appropriate treatment. This will result in direct accurate treatment and therefore could reduce the BCC recurrence rate. Therefore, the purpose of this study was to define confocal features characteristic of subtypes of BCC to allow a non-invasive diagnosis.

We evaluated the RCM features of 41 histopathologically confirmed BCCs. Importantly, RCM features of nBCC, mnBCC and sBCC were defined. It was demonstrated that tumor nests (aggregations of basaloid cells) with peripheral palisading, branch-like structures, fibrotic septa and increase in vascular diameter were the main characteristic RCM features for nBCC and mnBCC. The size, shape and location of the tumor nests allows further distinction between nBCC and mnBCC. Solar elastosis and the location of the tumor nests just below or in connection with the basal cell layer characterize sBCC. Although this study shows that RCM might allow in vivo diagnosis of nBCC, mnBCC and sBCC, RCM features for iBCCs still need to be defined. It was impossible to define RCM features for iBCC based on the included mixed-type BCC with an iBCC component in the deeper dermis. Unfortunately, we were not able to include single-type iBCCs. However, the incidence of so few iBCCs fits the epidemiology of BCC in general.

In addition to the described RCM features for the subtypes of BCC, we found large highly reflective plump bright cells in a part of the evaluated BCCs. These plump bright cells might be inflammatory cells. However, these cells were larger and different in shape compared to the already described inflammatory cells in BCC like lymphocytes and dendritic cells. These cells might be melanophages. With RCM these cells appear as bright, plump, oval or star-shaped cells with no visible nucleus and illdefined edges. This RCM description corresponds highly to the observed plump bright cells surrounding the BCC nests but should be confirmed by immunohistochemical staining.

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References


2.2

Prospective differentiation of clinically difficult to distinguish nodular basal cell carcinomas and intradermal nevi by reflectance confocal microscopy

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Abstract

Background: Clinical differentiation between a nodular basal cell carcinoma (nBCC) and a benign intradermal nevus can be difficult. Even with additional dermoscopic evaluation, a correct diagnosis may be difficult. Currently, histopathological examination of a biopsy is the gold standard to differentiate between these lesions. However, this is an invasive technique and sampling errors can occur. In vivo reflectance confocal microscopy (RCM) is a non-invasive technique to evaluate a skin lesion at a microscopic level. RCM features of nBCCs and intradermal nevi have been described in research setting. However, the use of RCM for prospective differentiation between difficult to diagnose nodules into nBCCs and intradermal nevi in clinical practice has not been demonstrated yet.

Objective: In this study, we aim to address a common clinical scenario; to differentiate clinically and dermoscopically difficult to distinguish nodules, into nBCCs and intradermal nevi by RCM.

Material and methods: Six patients with clinically and dermoscopically difficult to distinguish nodular skin lesions were evaluated by RCM to differentiate prospectively between nBCCs and intradermal nevi. In five out of six cases, a 3-mm punch biopsy was obtained to confirm the RCM diagnosis.

Results: Observed RCM features that allowed differentiation between nBCCs and intradermal nevi were the dermal-epidermal junction patterns, the appearance of the nests and the degree of vascularization.

Conclusions: This case series study demonstrates the value of non-invasive in vivo RCM imaging in routine patient care, with respect to the prospective diagnosis of clinically difficult to distinguish nBCCs and intradermal nevi. Subsequently, biopsies of benign lesions in cosmetic areas could be avoided.

Introduction

Clinically, it can be challenging to distinguish between nodular basal cell carcinomas (nBCCs) and intradermal nevi. A nBCC appears as a shiny, pearly, sometimes erythematous nodule with telangiectasias. These clinical features can mimic intradermal nevi. Correct differentiation between these two kinds of lesions is necessary, because they require a different treatment approach. In case of a nBCC, a biopsy and excision of the lesion is required, whereas these procedures are unnecessary in benign intradermal nevi. Dermoscopy is an additional tool, which may help to differentiate between these equivocal nodular lesions. Besides the well-known specific features for BCCs and intradermal nevi, they may share some dermoscopic characteristics. In addition, in many nodular lesions no specific dermoscopic features may be seen, often because of the elevated shape of these lesions. This clinical and dermoscopic limitations may often result in obtaining a biopsy to determine the correct diagnosis.

In vivo reflectance confocal microscopy (RCM) is a useful non-invasive technique to evaluate a lesion at a microscopic level, in a horizontal plane, without obtaining a biopsy. RCM features and terminology for nodular lesions like BCCs and nevi have been described. However, the use of RCM for prospective differentiation between difficult to diagnose nodules into nBCCs and intradermal nevi in clinical practice has not been demonstrated yet. Therefore, in this study, we aim to address a common clinical scenario; to differentiate between clinically and dermoscopically difficult to distinguish nBCCs and intradermal nevi, with the additional use of non-invasive RCM.

Materials and methods

Patients

Six patients with nodular lesions, clinically and dermoscopically suspect for nBCCs and intradermal nevi were included. They were referred from the outpatient Department of Dermatology, Radboud University Medical Center, Nijmegen, The Netherlands. The lesions were located in the face or at the chest of the patients.

Images

Prior to RCM imaging, clinical pictures of the lesions were taken with a digital camera (Nikon D 200, Nikon, Tokyo, Japan) and dermoscopic pictures were obtained with a Vivacam Macro Camera (Lucid Inc., Rochester, NY, USA).

RCM evaluation

The commercially available VivaScope 1500 system (Lucid Inc.) was used to obtain the RCM images. The RCM system has been extensively described elsewhere. The imaging was performed according to a standardized protocol. All different levels of the skin
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(stratum corneum, stratum granulosum, stratum spinosum, dermal–epidermal junction (DEJ) and papillary dermis) were imaged by a horizontal mapping of 4 mm x 4 mm (Vivablock). When indicated, Vivastacks (vertical mapping with steps of 4.5 µm) were made from the stratum corneum until the papillary dermis. At the DEJ and dermis, vascularization was captured in several movies. Two RCM users evaluated the lesions and made an in vivo diagnosis based on the obtained confocal features.

Histopathological evaluation
After the RCM evaluation, a 3-mm biopsy (under local anaesthesia of 1% xylocaine/adrenaline) was obtained in five of six lesions and evaluated by a dermatopathologist. The skin samples were processed by a standard protocol. Eventually the sections were haematoxylin-eosin stained for assessment of histopathological characteristics. The RCM diagnoses were compared to the histopathological diagnosis of the dermatopathologist.

Results
Case 1
A 61-year-old woman, frequently sun exposed, was referred to our outpatient clinic. During the last 2 years, a slow growing lesion on the right nasolabial fold had developed. Clinically, a well-demarcated, lenticular, pinkish nodule with telangiectasia was seen (Figure 1a). Dermoscopy revealed telangiectasia (Figure 1b). Based on clinical and dermoscopic examination, it was not possible to differentiate between a nBCC or intradermal nevus. RCM imaging showed architectural disarray of the epidermis and a non-specific architecture of the DEJ. Round to oval nests of basaloid cells, varying in size, were observed at the dermal level (Table 1). Within these nests, peripheral palisading and branch-like structures were present. The nests were surrounded by a cleft, fibrosis and blood vessels with enlarged diameter. These RCM findings suggested a nBCC with micronodular component (Figure 2a). Histopathology demonstrated large nests of basaloid cells with peripheral palisading and surrounding cleft, corresponding to a nBCC. Re-evaluation of the biopsy specimen revealed also small basaloid nests, as observed with RCM, indicating a mixed-type BCC with a nodular and micronodular component (Figure 2b). The lesion was surgically excised.

Case 2
A 48-year-old woman known with nevoid basal cell carcinoma syndrome, also known as Gorlin–Goltz syndrome,12 visited our department. During this visit, multiple lesions suspicious for nBCCs or intradermal nevi were found. We only describe a prominent lesion on the left nasolabial fold, which appeared as a skin-coloured nodule with telangiectasias (Figure 1c). Dermoscopy showed small telangiectasia, but could not discriminate between an intradermal nevus or nBCC (Figure 1d). RCM imaging of the epidermis revealed mild keratinocyte atypia and spongiosis (Table 1). At dermal level, one large round nest of

Figure 1. Clinical and dermoscopic images (VivaCam) of the evaluated lesions. Case 1: a) Clinical image of the well-demarcated, lenticular, pinkish nodule with telangiectasia, on the right nasolabial fold. b) Dermoscopic image revealing telangiectasia.
Figure 1 Continued. Case 2: c) A skin- coloured nodule with telangiectasias in the left nasolabial fold. d) Small telangiectasia were seen with dermoscopy. Case 3: e) A periorcular located erythematous, partly pigmented nodule with telangiectasia. f) Corresponding dermoscopic picture showing telangiectasia and dark brown dots. Case 4: g) Clinical image of the perioral located lesion, showing a pearly erythematous nodular lesion with telangiectasia. h) Dermoscopic image exhibiting cobblestone globules. Case 5: i) A shiny, pearly nodule with telangiectasia located on the chest. j) Dermoscopy revealed telangiectasia. Case 6: k) A skin-coloured perioral located nodule. l) Dermoscopic image showing sparse telangiectasia.

tightly packed basaloid cells was observed (Figure 2c). Within this nest, enlarged blood vessels were seen in real time. Furthermore, dermal solar elastosis and fibrosis were noted. The large nest visualized by RCM corresponded to the large nest of basaloid cells with peripheral palisading seen in conventional histopathology (Figure 2d). Based on these findings, the lesion was diagnosed as a nBCC and was surgically excised.

Case 3
A 47-year-old man without a history of non-melanoma skin cancer (NMSC), had an erythematous, partly pigmented, nodule with telangiectasias (Figure 1e). The lesion was located in the periocular region for at least 20 years. However, the patient noticed some change in appearance during the last year, the lesion had grown slowly and bled sometimes. On dermoscopy this lesion showed brown dots and telangiectasia (Figure 1f). RCM imaging was performed to non-invasively evaluate the lesion. The epidermis was characterized by mild keratinocyte atypia and spongiosis. Furthermore, the DEJ revealed a non-specific pattern. Large and small nests of basaloid cells appeared as a bunch of grapes and were found in the upper and deeper dermis (Figure 2e, Table 1). Peripheral palisading and branch like structures were present within these nests, and the nests were surrounded by a cleft, fibrosis and blood vessels with an increased diameter (Figure 2e). These RCM findings were in favor of a mixed-type BCC with nodular and micronodular component. This diagnosis was confirmed by conventional histopathology (Figure 2f). The lesion was surgically excised.

Table 1 Scored RCM features of the imaged lesions

<table>
<thead>
<tr>
<th>Features</th>
<th>nBCCs</th>
<th>Intradermal nevi</th>
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<tbody>
<tr>
<td></td>
<td>Case I</td>
<td>Case II</td>
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<tr>
<td>Epidermis</td>
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<tr>
<td>Regular honeycomb pattern</td>
<td>-/+</td>
<td>-/+</td>
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<tr>
<td>Regular cobblestone pattern</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Mild keratinocyte atypia epidermis</td>
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<td>+</td>
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<tr>
<td>Mild spongiosis spinosum</td>
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<td>+</td>
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<tr>
<td>Dermoeal-dermal junction</td>
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<tr>
<td>Ringed pattern</td>
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<td>-</td>
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<td>Clod pattern</td>
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<tr>
<td>Nonspecific pattern</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Edged papillae</td>
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<td>-</td>
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<tr>
<td>Non-edged papillae</td>
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<tr>
<td>Dermis</td>
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<tr>
<td>Nests containing cellular structures</td>
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<td>+</td>
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<tr>
<td>Large (&gt;300µm)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Small (&lt;300µm)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Round- oval shaped</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bunch of grape-like shaped</td>
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<td>-</td>
</tr>
<tr>
<td>Dense</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sparse</td>
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<td>-</td>
</tr>
<tr>
<td>Branch-like structures</td>
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<td>-</td>
</tr>
<tr>
<td>Peripheral palisading</td>
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<td>-</td>
</tr>
<tr>
<td>Cleft</td>
<td>+</td>
<td>-/+</td>
</tr>
<tr>
<td>Fibrosis surrounding nests</td>
<td>+</td>
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<tr>
<td>Blood vessels close to nests</td>
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<td>+</td>
</tr>
<tr>
<td>Diameter blood vessels</td>
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<tr>
<td>Solar elastosis</td>
<td>-</td>
<td>+</td>
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<td>Inflammatory cells</td>
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-, absent; -/+, mildly present; +, present; ++, increased; ++++, more increased; nBCCs, nodular basal cell carcinomas; RCM, Reflectance confocal microscopy.
Case 4
An 80-year-old woman visited our department after treatment of a previous nBCC. During this follow-up visit, a pearly erythematous nodular lesion with telangiectasias was seen in the right perioral region (Figure 1g). Dermoscopy revealed cobblestone globules (Figure 1h). RCM imaging of the epidermis showed a regular honeycomb pattern and at the upper papillary dermis several small dense (Figure 2g) and sparse nests were found (Table 1). RCM revealed the dermal nests, some of them were surrounded by small blood vessels. These features were suggestive for a benign intradermal nevus. Histopathology showed dermal nests of non-atypical melanocytes (Figure 2h), corresponding to the dermal nests seen with RCM and fitting to a diagnosis of intradermal nevus. As this lesion was benign, no further treatment was needed.

Case 5
A 34-year-old woman, with severe sunburns during childhood, had a shiny, pearly nodule with telangiectasias on her chest, suspicious for either a nBCC or an intradermal nevus (Figure 1i). Telangiectasias were seen with dermoscopy. Clinical and dermoscopic evaluation were not conclusive for differentiation between a nBCC or an intradermal nevus (Figure 1j). RCM imaging of the epidermis showed a regular honeycomb pattern. In addition, at the level of the DEJ, a clod pattern with non-edged papillae was observed (Table 1). The clod pattern is characterized by dense packed aggregates, formed by melanocytes within the dermal papillae. Small and large, dense and sparse nests (Figure 2i) were visible in both the upper and deeper dermis. These dermal nests were surrounded by blood vessels. Furthermore, solar elastosis was present at the level of the dermis. These RCM features suggested a benign nevus. The diagnosis of an intradermal nevus was confirmed by histopathological examination demonstrating dermal nests of non-atypical melanocytes (Figure 2j). No further treatment was performed.

Case 6
A 47-year-old woman with a history of NMSC, was referred to our outpatient clinic, for the presence of a skin-coloured nodule (Figure 1k), located in the perioral area. On dermoscopy sparse telangiectasias were visible (Figure 1l). RCM examination showed mild keratinocyte atypia and spongiosis of the epidermis, but overall it showed a regular honeycomb pattern. At the basal layer, a cobblestone pattern was visible (Table 1). The DEJ was characterized by a regular ringed pattern with edged papillae (Figure 2k). Small, dense, round to oval dermal aggregates surrounded by small capillaries were seen in the papillary dermis. Based on these RCM features and the RCM learning curve, the lesion was diagnosed as an intradermal nevus. No biopsy was obtained and no further treatment was performed.

Figure 2. Reflectance confocal microscopy (RCM) images (en face) of discriminating features and corresponding haematoxylin-eosin (HE) stained transversal tissue slides of nodular basal cell carcinomas (nBCCs) (a–f), and intradermal nevi (g–l). Case 1. a) RCM image demonstrates two small nests of basaloid cells (white asterisks). Within these nests, peripheral palisading and branch-like structures are present. The nests are surrounded by a cleft, fibrosis and blood vessels with enlarged diameter (white arrow). b) Corresponding HE stained tissue section showing a large nodus of basaloid cells with peripheral palisading and surrounding cleft. The micronodular nests are depicted by the black arrows. Case 2:
Dense nests are round to oval nests with dense clusters of monomorphic individual nevus cells (white arrows). The nests are surrounded by a cleft and blood vessels with enlarged diameter (white arrow). Highly reflective branches and peripheral palisading are recognizable within these nests. The nests are composed of aggregates of well-demarcated round to oval nevus cells with dark nucleus and reflecting cytoplasm in which individual nevus cells (white arrow) are easily to discern.13, 14

Histopathology shows dermal nests of non-atypical melanocytes (black arrows). The line indicates the level at which the en face oriented RCM image is obtained. Case 5. i) These round to oval structures visualized by RCM indicate sparse dermal nests (75 µm depth). The sparse clusters are nests composed of aggregates of well-demarcated round to oval nevus cells with dark nucleus and reflecting cytoplasm in which individual nevus cells (white arrow) are easily to discern.13, 14 j) Corresponding HE stained section showing dermal nests of non-atypical melanocytes and clusters of melanin (black arrow). Case 6. k) With RCM, bright round edged papillae (white arrows) are observed at the dermal-epidermal junction. l) Representative transversal histopathology of an intradermal nevus reveals dermal papillae (black arrows) that contain melanin in the basal layer of the epidermis, corresponding to the rings seen with RCM in (k). The line indicates the level at which the en face oriented RCM image is obtained.

Summary of cases

We observed several differences and similarities in RCM features for nBCCs and intradermal nevi. The imaged intradermal nevi showed a honeycomb pattern and minimal alterations in architecture of the epidermis. In contrast, the evaluated BCCs revealed abnormalities of the epidermis characterized by mild keratinocyte atypia and spongiosis. None of the BCCs showed a specific pattern of the DEJ. The DEJ of the intradermal nevus revealed a ringed or clod pattern with (non)edged papillae. The appearance of BCC nests compared to nevus nests, located at dermal level, was different. Dermal nests of nevus cells were characterized by dense or sparse aggregates. Nests of basaloïd cells were visualized as tightly packed cells, with peripheral palisading, surrounded by a cleft and fibrosis. Furthermore, BCCs showed a prominent vascularization with enlarged blood vessels, surrounding or within the nests of basaloïd cells. Although the imaged intradermal nevus revealed some blood flow, the perilesional blood vessels were less prominent compared to BCC.

Figure 2. Continued. c) Confocal image of the dermis (100 µm depth) demonstrating one large nest of basaloid cells (white asterisk). d) HE stained tissue section showing one large nodus of basaloid cells (black asterisk). Case 3. e) Bunch of grapes-like shaped nests of basaloid cells (white asterisk) visualized by RCM at dermal level (100 µm). Highly reflective branches and peripheral palisading are recognizable within these nests. The nests are surrounded by a cleft and blood vessels with enlarged diameter (white arrow). f) Corresponding HE stained sections showing nests of basaloid cells (black asterisk) with peripheral palisading in the upper dermis. Case 4. g) RCM imaging shows small, round to oval, dermal nests representing dense clusters of nevus cells (white arrows). Dense nests are round to oval nests with dense clusters of monomorphic individual nevus cells which are hardly distinguishable. Some of the dense nests are more reflective than others. 13, 14

h) Histopathology shows dermal nests of non-atypical melanocytes (black arrows). The line indicates the level at which the en face oriented RCM image is obtained. Case 5. i) These round to oval structures visualized by RCM indicate sparse dermal nests (75 µm depth). The sparse clusters are nests composed of aggregates of well-demarcated round to oval nevus cells with dark nucleus and reflecting cytoplasm in which individual nevus cells (white arrow) are easily to discern. 13, 14 j) Corresponding HE stained section showing dermal nests of non-atypical melanocytes and clusters of melanin (black arrow). Case 6. k) With RCM, bright round edged papillae (white arrows) are observed at the dermal-epidermal junction. l) Representative transversal histopathology of an intradermal nevus reveals dermal papillae (black arrows) that contain melanin in the basal layer of the epidermis, corresponding to the rings seen with RCM in (k). The line indicates the level at which the en face oriented RCM image is obtained.

Discussion

Clinical differentiation between a nBCC and a benign intradermal nevus can be difficult.1 Even with additional dermoscopic evaluation, a correct diagnosis may be difficult. Currently, histopathological examination of a biopsy is the gold standard to diagnose a BCC. However, this technique has disadvantages, as it is an invasive procedure and selects only a small part of the lesion, which can cause sampling errors.15, 16 As excisions margins differ between subtypes of BCC, this may lead to inadequate treatment.

In our outpatient clinic, RCM appears to be of additional value in establishing the correct diagnosis of nBCCs and intradermal nevi. Most of the observed RCM features were in line with features described in literature and correlate well with the commonly known histopathological characteristics. Described RCM features of BCC are a honeycomb or cobblestone pattern, mild disarray of the epidermis, a cauliflower or non-specific architecture of the DEJ, inflammatory infiltrates and prominent vascularity with enlarged vessels.17 Furthermore, tumor nests of basaloid cells with peripheral palisading, bright filaments and a surrounding cleft are the most specific RCM features for BCC.18 Well-known RCM features for intradermal nevi are a honeycomb or cobblestone pattern, ringed-, clod-, meshwork or non-specific architecture of the DEJ, with or without junctional nests or sheet-like structures, and dermal dense and – sparse nests. 9, 10, 17

In contrast to literature we did not observe a specific pattern of the DEJ in nBCCs. In literature, in many BCCs the DEJ has been described as a cauliflower architecture, corresponding to solid units of tumor cells, appearing as hypo-reflective aggregates surrounded by dark areas.4, 17 Vascularization has been visualized by RCM in only 20% of the nevi. However, in intradermal nevi it was a more common phenomenon (36%).19 In contrast, we observed vascularization in all three evaluated intradermal nevi.

The learning curve of RCM is of importance for a correct diagnosis. In experienced hands, RCM provides a non-invasive diagnosis within 5–10 min, which is significantly shorter than the total processing time of a biopsy. In all six cases, the correct diagnosis was made by means of the helpful non-invasive RCM. In one of the BCCs, a sampling error was avoided, because revision of a biopsy revealed also a micronodular component (besides the nodular aspect), as noticed with RCM. Detection of the micronodular component had therapeutic consequences, because a micronodular BCC is excised by a larger margin than a nBCC. In one intradermal nevus, RCM imaging avoided a biopsy of a benign lesion in the face. Noteworthy, pigmented BCCs and nevoid melanoma might be pitfalls of differentiation between nBCCs and intradermal nevi by RCM. However, these diagnosis were not within the scope of this study.

Overall, this case-series study demonstrates that in routine patient care, RCM allows prospective in vivo differentiation between clinical equivocal nodular lesions e.g. nBCCs and intradermal nevi. Subsequently, biopsies of benign lesions in cosmetic areas could be avoided. Future studies have to explore the sensitivity and specificity of this clinical RCM application.
References

2.3 Reflectance confocal microscopy: non-invasive distinction between actinic keratosis and squamous cell carcinoma
Abstract

Background: Early recognition of squamous cell carcinoma (SCC) is difficult. Non-invasive reflectance confocal microscopic (RCM) imaging of the skin is a promising diagnostic technique. Although several RCM features for SCC and actinic keratosis (AK) have been described, it is not determined whether RCM has the ability to distinguish between SCC and AK.

Objective: To determine in vivo reflectance confocal microscopic features that are specific for making a distinction between AK and SCC.

Methods: In 24 patients, 30 lesions clinically suspicious for AK or SCC were selected for RCM imaging. Following the imaging procedure, a 3-mm skin biopsy was obtained for confirmation of the histopathological diagnosis. Two observers evaluated the RCM images according to a literature based list of RCM features. The obtained data were evaluated by an univariate and forward multivariate logistic regression analysis, kappa analysis and independent T-test.

Results: The univariate logistic regression showed statistically significant odds ratios for several RCM features, including architectural disarray in the stratum granulosum, architectural disarray in the spinous layer and nest-like structures in the dermis. The forward multivariate logistic regression analysis showed that the combination of these features increased the ability to make the correct diagnosis of a AK and SCC non-invasively. The interobserver agreement between a starting and an experienced RCM observer ranged from poor to no agreement.

Conclusion: This study revealed specific RCM features that can distinguish between AK and SCC, stimulating further prospective large cohort research in this field. This will result in correct, efficient and adequate diagnosis and treatment of clinically difficult to distinguish AK and SCC lesions.

Introduction

Skin cancer is the most commonly diagnosed cancer in the Caucasian population, with rapid further increasing incidence rates. Squamous cell carcinoma (SCC) and basal cell carcinoma are considered as non-melanoma skin cancers (NMSC). The incidence ratio between those two NMSC types is approximately 1:4-5. Despite the lower frequency, SCC accounts for the majority of NMSC related metastatic disease, making early recognition important.

SCC arises out of epidermal keratinocyte dysplasia. These atypical keratinocytes penetrate the basal membrane in order to involve the dermis and deeper tissues. Actinic keratosis (AK) are commonly considered as pre-malignant skin lesions, which act as precursor to SCC. It is demonstrated that up to 20% of all AK lesions can progress to invasive SCC. However, it is not possible to predict which lesion is at risk. The development of AK lesions is induced by ultraviolet radiation, causing damage to keratinocytes and their proliferation. In contrast to SCC, the basal membrane is not disrupted in AK lesions.6-4

The diagnosis of AK is mainly made upon clinical evaluation. In contrast, a lesion clinically suspected for SCC is confirmed by histological evaluation of a skin biopsy. However, the clinical distinction between AK and SCC can be difficult and is not always reliable. Dermoscopy can be useful in determining the diagnosis non-invasively. Although, in SCC and SCC in situ, glomerular or dotted vessels are often visible, the absence of these vessels will not exclude the presence of a SCC. In addition, there may be some overlay between dermoscopic features of AK and SCC. Lastly, SCC is often difficult to visualize by dermoscopy because the scaly surface might obscure the underlying morphology. Therefore, routine histopathology remains the gold standard, although this entails patient discomfort, time and expenses. Moreover, the feasibility of obtaining biopsies from affected and surrounding skin sites is sometimes limited and can result in a sampling error. For these reasons, the interest in development of non-invasive diagnostic methods to distinguish between AK and SCC is increasing.

Reflectance confocal microscopy (RCM) is a non-invasive technique for in vivo imaging of the skin that uses near-infrared laser light. This technique produces horizontal images of the skin in shades of grey, with a resolution comparable to conventional histology. Non-invasive RCM is painless, can evaluate a larger area or the whole tumor, can image the exact same location over time and will not induce any kind of skin damage or inflammatory response. Further, artefacts caused by tissue processing during histopathological assessment can be avoided.

Currently, RCM is used for several dermatological purposes such as diagnosing and monitoring of inflammatory skin diseases, melanoma and NMSC, including SCC and its precursor AK. Several RCM features for AK and SCC have been described previously. However, to the best of our knowledge, there are no studies that have determined the ability of RCM to distinguish between AK and SCC in vivo. Therefore, the aim of this study
CHAPTER 2 NON-MELANOMA SKIN CANCER

is to determine, based on statistical evaluation, whether there are RCM features that are specific for making an in vivo distinction between AK and SCC.

Materials and methods

Subjects

In 24 patients (12 men and 12 female), lesions clinically suspicious for AK or SCC were included for RCM imaging. The age of the patients ranged from 53 to 80 years, with a mean age of 67 years. In 19 patients, a history of NMSC was documented. Four of these patients used chronic immunosuppressive drugs after kidney transplantation and one patient was treated with radiotherapy. Within all patients, the skin type varied between I and III, according to the Fitzpatrick scale. The patients were recruited from the Department of Dermatology, Radboud University Medical Center, Nijmegen, The Netherlands. Skin lesions with significant hyperkeratosis, extensive crusts, ulcerations or lesions located on body sites that were inaccessible for the RCM probe were excluded. A small control group of two subjects without a skin condition was included to compare vascular RCM features. This study was approved by the local medical ethics committee and was conducted according to the principles of the Declaration of Helsinki.

RCM imaging and analysis

For navigation during RCM imaging, pictures with a lower quality than dermoscopy were taken using a Vivacam macro camera (Vivacam; Lucid Inc., Rochester, NY, USA). In vivo RCM imaging was performed using the Vivascope 1500 system (Lucid Inc.). A detailed description of this technique has been published previously. Vertical mapping (Vivastack) was performed by capturing a series of images of 0.5 x 0.5 mm with steps of 4.5 µm in depth. The mapping started at the stratum corneum until the papillary dermis. Horizontal mapping of 4 x 4 mm (Vivablock) were made at different levels of the skin. In this study, the first appearance of nucleated cells, independent of the cell size and shape, was considered as the granular layer. Since the granular layer is only a few cell layers thick, two steps in depth below this point was considered as the spinous layer. In most lesions, a movie was made at the level of the dermal-epidermal junction in order to visualize capillary blood flow. Images were obtained using Vivascan 7.0 software (Lucid Inc.). For image analysis, a list of diagnostic RCM features for AK and SCC was composed according to literature (Table 1). RCM images were retrospectively evaluated for these features by an experienced RCM user (2.5 years), and a starting RCM user who was instructed in the basic interpretation of RCM imaging for approximately 2 weeks. Both observers were not blinded for the final diagnosis and evaluated the lesions systematically for the presence or absence of individual RCM features. Further, the mean blood vessel diameter and number of vessels per confocal image (0.5 mm x 0.5 mm) were determined for both AK and SCC lesions. These measures were compared to the control group. An increased vascular

<table>
<thead>
<tr>
<th>Table 1</th>
<th>List of RCM features for actinic keratosis (AK) and squamous cell carcinoma (SCC) with the description used in the evaluation and analysis of this study. This list was composed according to literature.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCM features</td>
<td>Described for AK</td>
</tr>
<tr>
<td>Stratum corneum</td>
<td></td>
</tr>
<tr>
<td>SC disruption, detached corneocytes</td>
<td>x</td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td>x</td>
</tr>
<tr>
<td>Parakeratosis</td>
<td>x</td>
</tr>
<tr>
<td>Orthokeratosis</td>
<td>x</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>x</td>
</tr>
<tr>
<td>Stratum granulosum</td>
<td></td>
</tr>
<tr>
<td>Normal honeycomb pattern</td>
<td>x</td>
</tr>
<tr>
<td>Atypical honeycomb pattern</td>
<td>x</td>
</tr>
<tr>
<td>Architectural disarray</td>
<td>x</td>
</tr>
<tr>
<td>Cellular and nuclear pleomorphism</td>
<td>x</td>
</tr>
<tr>
<td>Targetoid cells 1</td>
<td>x</td>
</tr>
<tr>
<td>Targetoid cells 2</td>
<td>x</td>
</tr>
<tr>
<td>Multinucleated keratinocytes</td>
<td>x</td>
</tr>
<tr>
<td>Stratum spinosum</td>
<td></td>
</tr>
<tr>
<td>Atypical honeycomb pattern</td>
<td>x</td>
</tr>
<tr>
<td>Architectural disarray</td>
<td>x</td>
</tr>
<tr>
<td>Cellular and nuclear pleomorphism</td>
<td>x</td>
</tr>
<tr>
<td>Targetoid cells 1</td>
<td>x</td>
</tr>
<tr>
<td>Targetoid cells 2</td>
<td>x</td>
</tr>
<tr>
<td>Multinucleated keratinocytes</td>
<td>x</td>
</tr>
<tr>
<td>Spongiosis</td>
<td>x</td>
</tr>
<tr>
<td>Exocytosis</td>
<td>x</td>
</tr>
<tr>
<td>Dermo-epidermal junction</td>
<td></td>
</tr>
<tr>
<td>Increased blood vessel dilatation</td>
<td>x</td>
</tr>
<tr>
<td>Increased number of blood vessels</td>
<td>x</td>
</tr>
<tr>
<td>Increased capillary flow</td>
<td>x</td>
</tr>
<tr>
<td>Lymphocyte rolling</td>
<td>x</td>
</tr>
<tr>
<td>Dermis</td>
<td></td>
</tr>
<tr>
<td>Solar elastosis</td>
<td>x</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>x</td>
</tr>
<tr>
<td>Keratin pearl</td>
<td>x</td>
</tr>
<tr>
<td>Nest-like structure</td>
<td>x</td>
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</tbody>
</table>
dilatation was defined as a diameter of more than 5 µm and an increased number of blood vessels as more than 5 vessels per confocal image.

**Histopathology**

Following RCM imaging, punch biopsies with a diameter of 3-mm were obtained under local anaesthesia with 1% xylocaine/adrenaline. After 4 h fixation in formaldehyde, the skin samples were embedded in paraffin and thereafter sectioned and stained with haematoxylin-eosin (HE) for histopathological evaluation by a pathologist.

**Statistical analysis**

An univariate logistic regression analysis was performed on each individual RCM feature (predictor) for the diagnosis of AK, on the data set as obtained by the experienced RCM user. The same analysis was performed for SCC. The predictors were expressed in odds ratios (OR) with p-values. Predictors with a statistically significant OR (with a p-value < 0.05) were evaluated in a forward multivariate logistic regression analysis in order to make a prediction model for the diagnosis of AK and SCC. The difference in blood vessel diameter and number of vessels per RCM confocal in SCC and AK lesions were analyzed using an independent T-test. The interobserver agreement between the experienced observer and the starting RCM user was determined by kappa analysis (κ). The concordance was assessed by calculating the kappa value for each individual RCM parameter. A kappa value between 1 and 0.81 corresponded with an excellent interobserver agreement, values with high concordance included those features with κ = 0.8–0.61, moderate concordance with κ = 0.6–0.41, poor concordance with κ = 0.4–0.1 and a kappa of ≤0 corresponded to no interobserver agreement. All data analyses were conducted using computer software (SPSS Inc. version 20, Chicago, IL, USA).

**Results**

A total of 30 biopsy proven lesions were evaluated with RCM, of which 24 AK and 6 invasive non-pigmented SCC. The lesions were located on the head and neck area in 37% (n = 11), thorax in 27% (n = 8), upper extremities in 27% (n = 8) and lower extremities in 10% (n = 3).

**RCM features for distinction between AK and SCC**

Univariate logistic regression analysis resulted in statistically significant OR values for architectural disarray in the stratum granulosum, architectural disarray in the stratum spinosum and nest-like structures in the dermis (Table 2, Figure 1). The forward multivariate logistic regression analysis with these parameters showed that the presence of architectural disarray in the granular layer would result in a correct diagnosis in 84.6% of the SCC cases.

<table>
<thead>
<tr>
<th>RCM parameter</th>
<th>Predicated percentage correctly diagnosed SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG architectural disarray</td>
<td>84.6%</td>
</tr>
<tr>
<td>SS architectural disarray</td>
<td>88.5%</td>
</tr>
<tr>
<td>DERMS nest-like structure</td>
<td>88.5%</td>
</tr>
</tbody>
</table>

All selected parameters are shown in ascending order, according to p-value: SS, stratum spinosum; SG, stratum granulosum.

| Table 2 RCM features for the diagnosis of SCC and AK with significant odds ratios (p-value < 0.05) based on the univariate logistic regression analysis. Further, the prediction model for diagnosis of SCC based on these RCM features is shown by the forward multivariate logistic regression analysis. |
|----------------------|----------------------|----------------------|----------------------|
| RCM parameter | Odd ratio for diagnosis SCC | Odds ratio for diagnosis AK | p-value |
| SG architectural disarray | 24.0 | 0.042 | 0.013 |
| SS architectural disarray | 15.0 | 0.067 | 0.023 |
| DERMS nest-like structure | 11.0 | 0.091 | 0.029 |

Table 1 Continued.

- Detached corneocytes: White, highly refractive polygonal structure of approximately 30–40 µm in diameter in the stratum corneum.
- Hyperkeratosis: Thickening of the stratum corneum of more than 15 µm.
- Parakeratosis: Nucleated cells appearing as bright oval nuclei centrally within corneocytes in the stratum corneum.
- Orthokeratosis: Hyperkeratosis without parakeratosis.
- Inflammatory cells: Highly refractive structures of 8–10 µm in diameter.
- Normal honeycomb pattern: Uniform, regular spaced, broad keratinocytes forming a grid resembling a honeycomb.
- Atypical honeycomb pattern: Irregular shaped cells deviating from the normal honeycomb pattern.
- Architectural disarray: Severe disarranged epidermal pattern in which the honeycomb pattern is no longer visible. Cellular and nuclear pleomorphism, Variation in cellular and nuclear shape and size.
- Targetoid cells 1: Large cell with a bright centre and a dark peripheral halo.
- Targetoid cells 2: Large cell with a dark centre and a bright rim surrounded by a dark halo.
- Multinucleated keratinocytes: Large cells with tight aggregates of bright nuclei.
- Keratohyalin granules: Lactophenol cotton blue stained granules that stain dark blue-gray centrally within corneocytes.
- Keratin pearl: Whorl-shaped accumulation of keratin appearing as highly refractive, speckled structure in the stratum corneum.
- Multinucleated keratinocytes: Large cells with tight aggregates of bright nuclei.
- Keratin pearl: Whorl-shaped accumulation of keratin appearing as highly refractive, speckled structure in the dermis.
- Nest-like structure: Round, demarcated structures in the dermis that are often surrounded by fibrosis.
- Normal honeycomb pattern: Uniform, regular spaced, broad keratinocytes forming a grid resembling a honeycomb.
- Atypical honeycomb pattern: Irregular shaped cells deviating from the normal honeycomb pattern.
- Architectural disarray: Severe disarranged epidermal pattern in which the honeycomb pattern is no longer visible. Cellular and nuclear pleomorphism, Variation in cellular and nuclear shape and size.
- Targetoid cells 1: Large cell with a bright centre and a dark peripheral halo.
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- Multinucleated keratinocytes: Large cells with tight aggregates of bright nuclei.
- Keratohyalin granules: Lactophenol cotton blue stained granules that stain dark blue-gray centrally within corneocytes.
- Keratin pearl: Whorl-shaped accumulation of keratin appearing as highly refractive, speckled structure in the stratum corneum.
The combination of architectural disarray in the granular layer with architectural disarray in the stratum spinosum and/or dermal nest-like structures had a correct prediction of 88.5% of the SCC cases (Table 2). All other evaluated RCM features were not statistically significant and were therefore not able to distinguish between AK and SCC.

Increased vascularisation in SCC and AK lesions
Comparing healthy skin with AK and SCC lesions, the mean blood vessel diameter and number of blood vessels per RCM confocal were increased in both AK and SCC lesions. The blood vessel diameter and number of blood vessels were highest in SCC. However, the vascular differences between AK and SCC were not statistically significant when analysed by an independent T-test (Table 3).

Table 3 Blood vessel characteristics of healthy skin and patients with squamous cell carcinoma (SCC) and actinic Keratosis (AK)

<table>
<thead>
<tr>
<th></th>
<th>Mean blood vessel diameter $\pm$ SD (µm)</th>
<th>Mean number of blood vessel per $0.5 \times 0.5$ mm RCM confocal image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.26 ± 0.00</td>
<td>3.5 ± 2.1</td>
</tr>
<tr>
<td>AK</td>
<td>13.52 ± 9.89</td>
<td>7.8 ± 4.9</td>
</tr>
<tr>
<td>SCC</td>
<td>27.62 ± 32.25</td>
<td>86 ± 4.2</td>
</tr>
</tbody>
</table>

*p-value when compared to AK was 0.386  
*p-value when compared to AK was 0.739
The mean number of blood vessels per $0.5 \times 0.5$ mm RCM confocal image.

Interobserver agreement between independent observers
The interobserver agreement for the RCM parameters between an experienced and starting RCM user ranged from poor to no agreement. The highest concordance was reached for parakeratosis in the stratum corneum ($\kappa = 0.33$), architectural disarray in the stratum granulosum ($\kappa = 0.34$) and inflammation in the superficial dermis ($\kappa = 0.36$).

Discussion
RCM has been proven to be a useful, non-invasive tool for the in vivo diagnosis of melanocytic lesions and inflammatory skin conditions. Further, RCM knowledge and experience in the field of NMSC is increasing. Due to often clinical similar appearance, distinction between SCC and AK is still a challenge. Currently, the diagnostic distinction between these skin lesions, especially when solely based on clinical aspects, may not always be reliable. Whereas, obtaining biopsies is an invasive method and the feasibility is sometimes limited, mainly because of the risk of sampling errors. Therefore, the purpose of this study was to assess in vivo RCM features that are specific enough to make a distinction between AK and SCC using RCM as a non-invasive in vivo diagnostic method.

We demonstrated that, in clinically suspicious AK or SCC lesions, the presence of architectural disarray in the stratum granulosum in combination with architectural disarray in the spinous layer and/or tumor nest in the dermis were the main RCM features to
distinguish SCC from AK. This result is in agreement with other studies that found architectural disarray in the granular layer in SCC, while the stratum granulosum in AK showed either normal keratinocytes or an atypical honeycomb pattern. Therefore, architectural disarray in the spinous layer alone is not a good predictor for SCC. Although we found differences in the granular and spinous layer, it should be mentioned that it might be hard to make the distinction between the granular and spinous layer in vivo. A good definition of the layers and experience in RCM image analysis are required. The observed nest-like structures in the dermis correlate to aggregates of atypical keratinocytes corresponding to the diagnosis of invasive SCC. However, we also detected these nest-like structures in two AK lesions. This observation might have resulted from sampling error, whereby the biopsy was taken at a different site than where the nest-like structures were seen with RCM. This demonstrates the great advantage of RCM in evaluation of the total lesion, and therefore can prevent sampling errors.

Further, we found an increased mean vascular diameter and a larger number of vessels for SCC and AK. Our results are in line with other studies and can be explained by the high metabolic needs of a tumor, which leads to vascular dilatation and neovascularization.

AK can be categorized according to the Keratinocyte Intraepithelial Neoplasia (KIN) with subdivision into three histopathological grades. KIN I, the keratinocytic atypia is limited to the lower third of the epidermis, whereas in KIN II, the lower two-thirds of the epidermis is involved. In KIN III, including Bowens disease, cell atypia is found in the full thickness of the epidermis without infiltration of atypical cells into the dermis. Although Bowens disease develops as epidermal carcinoma in situ, it may progress into invasive SCC. Therefore, it would be very interesting and useful to evaluate in a larger cohort whether there are specific RCM features that allow distinction between KIN grades, Bowens disease and invasive SCC. Despite the fact that RCM has some limitations in depth, this study shows that there are epidermal RCM features that might allow in vivo distinction between clinical similar appearing AK and SCC lesions.

The overall poor to no interobserver agreement in this study showed that RCM features for AK and SCC were difficult to learn and assess for an inexperienced RCM user. This illustrates the learning curve, which evaluation of RCM images is associated with. In contrast, the interobserver agreement between experienced RCM users is higher. Horn et al. showed a moderate to poor interobserver agreement for RCM features of AK between two dermato-oncologists with previous experience in RCM. In addition, Ulrich et al. also found a higher concordance for AK features among two independent experts in the field of RCM. However, this kind of data are not available for the diagnosis of SCC lesions.

Dermoscopy is another commonly used non-invasive technique that improves the diagnostic accuracy of pigmented and non-pigmented skin lesions. Several dermoscopic features for AK and SCC are described, in which some can be observed by RCM. Fraga-Braghiroli et al. observed with RCM the appearance of round circles with a bright white rim at the level of the dermal-epidermal junction corresponding to the small brown circles that can be observed with dermoscopy in pigmented SCC. Unfortunately, we were not able to confirm this observation since this study only revealed non-pigmented lesions. In a larger prospective study, it would be interesting to include pigmented lesions as well. Although dermoscopy is a useful technique, it is not always conclusive due to similar appearing features between AK and SCC and the limitations of the surface examination. Especially in these cases real-time in vivo RCM, which can image the skin at morphological level until the papillary dermis, is of additional value.

It needs to be mentioned that for SCC, it remains difficult to include a large number of lesions. The often hyperkeratotic scale of a SCC is hard to evaluate with either dermoscopy or RCM, however, the major advantage of both techniques cannot be found in these clinical evident SCC. The major challenge is in the field of clinical similar appearing lesions and to distinguish between in situ SCC and invasive SCC. Our goal was to evaluate these similar appearing lesions, explaining the unequal number included AK and SCC.

In conclusion, this study revealed specific epidermal and dermal RCM features that can distinguish between AK and SCC in vivo. This stimulates further prospective, large cohort investigation in this field, which will contribute to development of protocols, resulting in correct, efficient and adequate diagnosis and treatment of clinically similar appearing AK and SCC. Furthermore, we have shown that extensive training and experience in RCM is required in order to correctly differentiate AK from SCC by RCM.
References

In vivo reflectance confocal microscopy: A useful tool to select the location of a punch biopsy in a large, clinically indistinctive lesion
Abstract
Reflectance confocal microscopy (RCM) is a non-invasive technique for in vivo imaging of the skin that allows evaluation of the total lesion area. This case report about a 66-year-old patient with a clinically indistinctive, previously treated erythematous lesion on the frontal part of the face demonstrates the use of RCM to select the proper biopsy location.

Introduction
Squamous cell carcinoma (SCC) is the second most common non-melanoma skin cancer after basal cell carcinoma (BCC).1, 2 Currently, diagnosis of a clinically suspect SCC or BCC is confirmed by histological evaluation of a skin biopsy obtained from the clinically most suspicious part of the lesion. However, a biopsy only represents a small part of the lesion. Especially in large lesions, this might result in a false-negative diagnosis or in missing a more aggressive part of the lesion.3, 4 Furthermore, clinical evaluation of previously treated and/or large lesions can be difficult, and in such cases, it is hard to determine the most suspect area for a biopsy.

Reflectance confocal microscopy (RCM) is a non-invasive technique for in vivo imaging of the skin that allows evaluation of the total lesion area.5 It is known that RCM can visualize non-melanoma skin cancer, melanoma and inflammatory skin diseases.6, 7 However, to the best of our knowledge, it is not described whether RCM might be used to select the location of a punch biopsy in a large and clinically indistinctive lesion.

Case Presentation
A 66-year-old patient with an erythematous lesion on the frontal part of the face was referred from the Department of Dermatology of the Bernhoven Hospital, Oss, The Netherlands. At this hospital, the lesion was treated with cryotherapy followed by Efudix. Unfortunately, both treatments were ineffective. A punch biopsy after these therapies revealed the presence of a superficial BCC and, therefore, the lesion was treated with photodynamic therapy. However, the lesion persisted and increased in size. The biopsies obtained after photodynamic therapy showed chronic inflammation and no residual tumor tissue. For further management, the patient was referred to the outpatient clinic of the Department of Dermatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. A clinical diagnosis of the erythematous lesion with a size of 2.3 × 2.1 cm was difficult due to the previously performed biopsies and treatments (Figure 1). This made it challenging to determine the most suspect area for a biopsy. Treatment by surgical excision without prior establishment of the diagnosis was unfavorable due to the size and the location of the lesion. Therefore, we used RCM for a non-invasive evaluation of the complete lesion to select the most suspect area for a biopsy.

The major part of the lesion showed RCM features corresponding to normal skin or actinic keratosis. More importantly, one small part showed prominent aberrant features: a disrupted stratum corneum, keratinocyte atypia, spongiosis, increased capillary blood flow, flattening of the dermal epidermal junction, solar elastosis and a nest of atypical cells (Figure 2). These features were suspect for either SCC or BCC. A 3-mm punch biopsy was taken from this part of the lesion. Histopathological evaluation of the biopsy showed the presence of a SCC with an invasive growth of 0.9 mm. The lesion was excised and
evaluation of the excision specimen showed multifocal actinic keratosis and two small foci displaying SCC with an invasive growth of 0.8 and 0.5 mm.

Figure 1 Clinical picture of an erythematous plaque located on the frontal area of the face. The lesion showed a heterogeneous appearance with erythema, mild induration and an indistinct margin. The lesion had a size of 2.3 × 2.1 cm and had been present for over 10 years.

Figure 2 Clinical and RCM images of the SCC with the corresponding histology. 

a) Clinically, an erythematous plaque with an irregular border was located on the frontal area of the face. Depigmentation was mainly found in the cranial part of the lesion.

b) Corresponding hematoxylin-eosin stained tissue section displaying an invasive SCC (black arrow).

c) RCM image showing flattening of the dermal epidermal junction. Islands of dermal cells (encircled area) are interspersed between epidermal areas.

d) Tumor nest consisting of atypical epidermal cells (white arrow) surrounded by fibrosis (asterisk).

e) Spongiosis and keratinocyte atypia, visualized as enlargement of the bright intercellular spaces and loss of the regular honeycomb pattern.

f) Keratinocyte atypia and streaming at the stratum spinosum.

g) Severe solar elastosis was seen in the dermis, visualized as coarse, highly reflective collagen bundles.
Conclusion

Although it is known that RCM can be helpful for the diagnosis of non-melanoma skin cancer, this case demonstrates the practical use of RCM as a tool to select the location for a punch biopsy in a large and clinically indistinctive lesion. In this case, the foci displaying SCC were only a small part of the total lesion area. Without RCM imaging prior to the biopsy, the most aggressive part of the lesion could have been missed. RCM allows evaluation of the total lesion area, which might reduce sample errors and delay in accurate diagnosis and treatment of large or clinically indistinctive lesions.

References

Dynamics in skin inflammation
3.1 Establishing the dynamics of neutrophil accumulation in vivo by reflectance confocal microscopy
Abstract

Reflectance confocal microscopy (RCM) is an imaging tool, which visualizes the epidermal skin layers in vivo with a cellular resolution. Neutrophil accumulation is a characteristic feature in psoriasis and is thought to play a role in the pathophysiology of psoriasis. Until now, imaging of neutrophil accumulation in vivo is not performed. We evaluated the dynamics of neutrophil migration in active psoriatic lesions by non-invasive RCM imaging. Additionally, we evaluated the time phasing and duration of neutrophil trafficking. We performed RCM imaging prior to the start of topical treatment and for seven consecutive days with a 24 h time interval at the Radboud University Medical Center, Nijmegen, the Netherlands. Twelve psoriatic lesions in three patients with a severe exacerbation of psoriasis were included. The four most active lesions were selected in each patient based on the highest degree of redness, induration and expansion in the previous 2 weeks. In all lesions, a cyclic pattern of neutrophil migration was observed, consisting of squirting papillae, transepidermal migration, accumulation in the stratum spinosum, accumulation in the stratum corneum and degeneration of the abscesses. The time interval of a neutrophil trafficking cycle was 5–7 days and showed a synchronic time phasing. This study is the first to establish the dynamics and time phasing of neutrophil migration in vivo in psoriatic lesions. Previously reported theories were confirmed by these novel in vivo data. RCM might distinguish between active or chronic psoriatic areas, which might contribute to new insights into the pathogenesis of psoriasis.

Introduction

Polymorphonuclear neutrophils (PMN) are pathognomonic for psoriasis. This accumulation of neutrophils occurs in four consecutive phases. First, the PMN migrate out of the capillaries inside the dermal papillae into the overlying thinned suprapapillary plate ('squirting of the papillae'). Subsequently, the PMN traffic upwards through the stratum spinosum. Then, PMN accumulate in the upper part of the Malpighian layer. The keratinocytes that surround the PMN accumulation degenerate and form a cavity. The accumulation of neutrophils within a spongiotic pustule is called 'spongiform pustule of Kogoj'. In the last phase, neutrophils migrate upwards in the direction of the stratum corneum and form micro-abscesses of Munro. These microabscesses are located almost exclusively within an area of parakeratosis and are typically located above dermal papillae.

Although the accumulation of neutrophils is a hallmark of psoriasis, spongiform pustules of Kogoj and microabscesses of Munro are reported in only 10% and 41% of psoriatic lesions, respectively. The presence during a limited time interval is consistent with a cyclic pattern. Intermittent squirting of dermal papillae and the alternating pattern of orthokeratosis and parakeratosis are additional histological clues pointing towards a cyclic and variable activity in a psoriatic lesion. The histological heterogeneity of psoriasis is consistent with the clinical variable pattern of psoriasis, consisting of remissions and exacerbations. Van de Kerkhof et al. related the presence of PMN to enlarging and very early lesions of psoriasis. Griffin et al. reported that the presence of neutrophils was associated with acute areas within a psoriatic lesion and that the absence of PMN was related to more chronic areas. Therefore, active or chronic lesions might be based on a different pathophysiological mechanism.

Currently, psoriasis is predominantly considered to be a disorder of innate immunity. The recruitment and activation of preferentially T helper 1 cells by natural killer T cells, dendritic cells and keratinocytes play a key role in the pathogenesis of psoriasis. Further, growth-related oncogene α and interleukin-8 are major chemoattractants that contribute to neutrophil diapedesis and migration. Production of interleukin-8 by keratinocytes is enhanced by interleukin-17, which is produced in large amounts by PMN. Interleukin-17 has a major role in the pathogenesis of psoriasis, because drugs targeting this cytokine are highly effective. Therefore, neutrophils are also thought to be important. Drugs causing a dose-dependent decrease in neutrophil counts are highly effective in the treatment for psoriasis. Additionally, established treatments for psoriasis (e.g. ultraviolet B phototherapy, methotrexate and topical corticosteroids) have a high variability in mode of action, but all inhibit PMN migration. Although neutrophils are thought to play an important role in the pathogenesis of psoriasis, signals for neutrophil diapedesis and migration in vivo are not yet fully understood.

Previously, the dynamics of PMN migration was studied by the application of leukotriene B4 (LTB4). LTB4 is a highly specific leukocyte chemoattractant that is naturally present in...
lesional psoriatic skin. Epicutaneous application of LTβ4 induces artificial intra-epidermal PMN accumulation with well-established time and phase intervals. However, to the best of our knowledge, no real-time in vivo studies have been performed so far that evaluate the dynamics of leucocyte accumulation in psoriasis or any inflammatory skin disease. The random pattern of neutrophil migration in psoriasis is another major difficulty in studying the dynamics of neutrophil trafficking. In vivo imaging of active lesions and imaging-guided biopsies could improve the understanding of the cyclic pattern of neutrophil migration.

Reflectance confocal microscopy (RCM) is a novel, non-invasive imaging technique. It can image the superficial layers of the skin with a resolution that is comparable to conventional light microscopy. Currently, this technique is mainly used to study melanocytic cells and lesions, but also to evaluate skin barrier function. Further, well-established histological features of psoriasis and other inflammatory skin lesions can be visualized with this technique, including characteristic PMN accumulations. It has been shown that these RCM images correlated highly with histology.

The previously reported theories about the phases of neutrophil trafficking and the fact that RCM might distinguish between active or chronic psoriatic areas might contribute to new insights into the pathogenesis of psoriasis. Therefore, the aim of this study was to evaluate the dynamics and the time interval of neutrophil migration by RCM, focussing on the previously described phases of neutrophil extravasation, epidermal migration, accumulation in the stratum spinosum (micropustules of Kogoj) and accumulation in the stratum corneum (microabscesses of Munro).

Materials and methods

Patients

We performed a pilot study in 12 psoriatic lesions of three patients with a severe exacerbation of psoriasis, because neutrophil migration is more prominent in patients with unstable psoriasis. In each patient, the four most active lesions were selected based on the most prominent redness, induration and expansion in the previous 2 weeks. The patients were recruited from the Department of Dermatology, Radboud University Medical Center, Nijmegen, the Netherlands. This study was approved by the local medical ethics committee and was conducted according to the principles of the Declaration of Helsinki. The patients gave written informed consent prior to inclusion. The lesions displayed characteristic erythematous plaques covering the trunk, arms, legs, intertriginous areas, face and scalp. In two patients, the lesions were located on the dorsal side of the forearm, and in one patient on the extensor surface of the upper leg. One of the patients was treated with dithranol, topical corticosteroids and methotrexate 22.5 mg a day. The other patients received topical treatment with dithranol for 4 days followed by tacrolimus, corticosteroids and coal tar 10%. The lesions selected for RCM imaging were not treated during the study.

Study Protocol

Transparent body charts were used to outline the lesions at baseline to allow colocalization during follow-up. RCM imaging was performed before the start of topical treatment (baseline) and for seven consecutive days, with a 24 h time interval. Before RCM imaging, a hyperkeratotic scale was carefully removed with a pair of tweezers to limit light scatter and optimize penetration depth. An area of 15 cm x 15 cm surrounding the lesions was outlined and patients were not allowed to use any psoriatic treatment on this area during the study, besides non-medicated topical dressings.

RCM Imaging

Confocal imaging was performed with a commercially available VivaScope 1500 System (Lucid Inc, Rochester, NY, USA). This confocal microscope comprises a near-infrared 830 nm low-power laser beam. RCM obtains horizontal (en face) images of the skin, in contrast to the vertical histological sections. Images were obtained using VivaScan 70 Software (Lucid Inc, Rochester, NY, USA). A more detailed description of the system has been published previously. RCM imaging was performed according to a standardized protocol. Horizontal mapping (4 mm x 4 mm) was performed at the level of the stratum corneum, stratum granulosum, stratum spinosum and at the dermal-epidermal junction (DEJ). Vertical mapping was obtained by capturing a series of images in depth (0.5 mm x 0.5 mm), with steps of 5 µm. The mapping started at the top of the stratum corneum until the DEJ. Additional images were made when neutrophil trafficking or accumulation was observed. Movies were made at the level of the DEJ to capture capillary blood flow and the presence or extravasation of neutrophils.

Biopsies: HE staining and immunohistochemistry

After RCM imaging, in four lesions 4-mm punch biopsies were taken at day seven for correlation to the RCM images. Local anaesthesia was used with 1% xylocain/adrenalin. The biopsies were fixed in formalin 10% and embedded in paraffin. Sections (5 µm) were haematoxylin–eosin (HE) stained for the assessment of histopathological features. Additionally, immunohistochemical staining was performed with monoclonal antibodies specific for CD3 (1:100 and 1:500) (clone F7.2.38, Abcam, Cambridge, UK) and elastase (1:10 000 and 1:50 000) (clone NPS7, Dako, Denmark). All sections were pretreated with peroxidase block (Dako, Copenhagen, Denmark). For the CD3 immunostaining, the sections were antigen-retrieved by boiling the sections in EDTA (pH 8.0, 0.5% Tween) for 10 min. All sections were air-dried and immersed in phosphate-buffered saline (PBS). For both stainings, this initial step was followed by incubation for 15 min with 1% bovine serum albumin (Organon Technika, Boksel, the Netherlands) in PBS. Next, overnight incubation with the primary antibody was performed. This step was followed by incubation with HRP anti-mouse Envision (DAKO, Copenhagen, Denmark) for 30 min. CD3 or elastase were visualized using 3,3′-diaminobenzidine (DAB). The sections were counter-
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stained with Mayer’s haematoxylin (Sigma, St Louis, USA) and mounted in Permount (BDH chemical, Poole, England). For better correlation with the en face RCM images, the biopsies were re-embedded in an orientation parallel to the skin surface (en face), sectioned (5 µm) and HE stained. The sections were photographed at a magnification of 10x, 20x or 40x using a microscope (Axioskop2 MOT, Zeiss, Oberkochen, Germany), digital camera (Axiocam MRC5, Zeiss) and AxioVision software (Zeiss).

Results

In all lesions, a cyclic pattern of neutrophil migration was observed, consisting of four consecutive phases. An overview of the RCM images of the neutrophil-trafficking cycle and the corresponding HE stained tissue sections is shown in Figures 1 and 2. The time interval of one neutrophil-trafficking cycle was 5–7 days. Within one lesion, a high variability was seen in the number and size of the areas where neutrophil trafficking occurred. Further, the neutrophil cycle showed a synchronous time phasing throughout the lesion. During follow-up, lesions either re-entered a neutrophil-trafficking cycle or showed normalization of the psoriatic histological features.

Neutrophil extravasation and trafficking

Prior to the start of the neutrophil-trafficking cycle, an increased capillary blood flow was seen inside the predominantly en face orientated capillaries at the DEJ. A large number of red blood cells and inflammatory cells were observed inside the capillary lumen by the quick movement of bright round cells. Neutrophil rolling and adhesion to the capillary endothelium was seen as a bright rim of dense, round to oval neutrophils adhering to the capillary wall (Figure 1, 2 and Video S1, which is available at http://onlinelibrary.wiley.com/doi/10.1111/exd.12345/suppinfo). Then, the PMN extravasated out of the capillaries into the overlying stratum spinosum. Notably, the PMN extravasated at the same time in a phasewise manner, representing squirting papillae. Subsequently, the PMN migrated through the stratum spinosum towards the stratum corneum. The trafficking was located just above the tips of the dermal papillae, which formed a column-like appearance of upwards migrating PMN (Figure 1).

Accumulation of neutrophils in the upper epidermal layers

About 24 h after extravasation, PMN accumulated at the level of the upper stratum spinosum and stratum corneum, and here, they formed micropustules of Kogoj and microabscesses of Munro, respectively. The micropustules of Kogoj were seen as an aggregation of bright cells surrounded by a dark acellular area. Based on histology, these cells corresponded to the presence of PMN. The surrounding stratum spinosum showed a flattened honeycomb pattern. The Munro microabscesses had a distinct morphology of large, polyform, very bright cells aggregating at the level of the stratum corneum. Based on histology, these cells represented PMN. In all lesions, parakeratosis was observed adjacent to the Munro microabscesses, which was shown as retention of bright nuclei in dark cornocytes.

Degeneration of abscesses

The abscesses started to degenerate about 24 h after their formation. An overview of the RCM images showing abscess degeneration is given in Figure 3. First, the PMN became less bright and had an increasingly vague cellular contour, resulting in a homogeneous grey area. About 24 h later, a dark area was seen with only a few scattered bright round PMN, representing further degeneration of the abscess.
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more polyform appearance. When the PMN accumulated and formed microabscesses of Munro, they had a prominent appearance of very bright, large cells with a polygonal shape.

Neutrophil morphology
Interestingly, the morphology of the PMN changed during the several phases of the neutrophil-trafficking cycle. When the PMN were located inside the capillaries or extravasated into the overlying stratum spinosum, they were highly refractive and had a round to oval shape. While trafficking though the stratum spinosum, the PMN showed a less round and

Biopsies: HE staining and immunohistochemistry
The HE stained tissue sections of all lesions displayed characteristic psoriatic features including parakeratosis, acanthosis and accumulation of PMN (Figure 2). The accumulations of neutrophils consisted of elastase positive cells and no CD3 positive cells. The psoriatic features in the HE stained en face tissue sections corresponded highly to the features found in the RCM images.

Figure 2 Transverse and corresponding en face histology of neutrophil accumulations in a psoriatic lesion. a) Tissue sections stained with haematoxylin-eosin (HE) (a1), CD3 (a2) and elastase (a3). The histology displays characteristic psoriatic features including parakeratosis and acanthosis. Accumulation of polymorphonuclear neutrophils (PMN) in the stratum corneum (asterisks b1) and upper stratum spinosum (arrow b2) is seen. These accumulations predominantly consist of elastase positive cells (a3) and almost no CD3 positive cells (a2). b) En face HE stained tissue slides corresponding to the levels indicated in a1. The PMN migrate upwards and accumulate at the stratum corneum. Further, adjacent parakeratosis is seen (asterisk, b1). Then, they migrated through the stratum spinosum (arrow), cluster together and form a large abscess (b5, with a higher magnification in b2). At the tips of the dermal papillae, the PMN extravasate out of the capillaries into the overlying stratum spinosum, representing squirting papillae (b3). There is leucocyte adhesion to the capillary endothelium (arrow b4) at the level of the dermal-epidermal junction. Many red blood cells can be seen in the capillary lumen, with high capillary blood flow during RCM imaging (b4).

Figure 3 Reflectance confocal microscopic (RCM) images of a Munro microabscess and micropustules of Kogoj at baseline and after 24, 48 and 72 h. a) RCM image at the level of the upper stratum spinosum. There are multiple accumulations of highly reflective polymorphonuclear neutrophils (PMN) surrounded by a dark area (white arrows). These accumulations are located above the tips of the dermal papillae forming circular patterns with a size comparable to the underlying papillae. The red asterisk represents an area of stratum spinosum surrounding the neutrophil accumulations. b) At the level of the stratum corneum, the PMN aggregate (white asterisk) and form abscesses (red asterisk). Stratum spinosum cells degenerate and individual cellular boundaries cannot be identified. c) After 24 h, the PMN cluster into one large abscess forming a microabscess of Munro. The PMN are very bright, large and display a polymorph appearance. d) After 48 h, the refractivity of the PMN diminishes and the individual cellular outlines cannot be observed. This results in a more homogeneous appearance, representing mild degeneration of the Munro abscess. e) After 72 h, only sparse PMN are seen (arrows), representing prominent degeneration of the Munro abscess.
Discussion

In the present study, we confirm for the first time the previously described theory of the dynamics of neutrophil migration in psoriatic lesions with in vivo data, using RCM. Further, we add novel data about the description of the duration and time phasing of neutrophil trafficking. In all lesions, a cyclic pattern of neutrophil migration was observed. This consisted of rhythmic neutrophil extravasation out of the dermal papilla, epidermal migration towards the stratum corneum, accumulation in the stratum spinosum, accumulation in the stratum corneum and phases of microabscesses degeneration. In the studied lesions, the time interval of a neutrophil-trafficking cycle was 5–7 days. Though, there could be a slightly different interindividual variability in a larger study population.

Pinkus et al. hypothesized that cycling squirting of dermal papillae and leucocyte migration and accumulation can be found in psoriatic lesions. They described that these events are limited in time and repeat themselves periodically. However, these ideas were only based on non-dynamic data. Our data confirm these hypotheses by in vivo data. Further, it is described that the squirting papillae reaction may result from focal and pulsed expression of the chemokines growth-related oncogene-α and interleukin-8. However, it was also suggested that complement factor C5a is more closely related to neutrophil chemotactic activity than interleukin-8. Interleukin-8 appears to be a proinflammatory cytokine involved in the induction of more baseline inflammatory changes.

Different pathophysiological mechanisms in acute and chronic lesions have been described by Terui et al. They report that psoriatic lesions are not uniform but heterogeneous, both clinically and histologically. They propose that in chronic lesions, a T-cell-mediated inflammation-sustaining loop maintains background ‘chronic’ inflammatory changes. In this loop, lymphokines produced by activated T cells induce proliferation of the epidermis. These stimulated keratinocytes release several cytokines, which in turn enhance the activation of T cells, forming a vicious cycle. In contrast, in acute lesions, island-like ‘acute’ inflammatory changes are induced. These are scattered over the chronic psoriatic plaques by a neutrophil-associated inflammation-boosting loop. In this loop, accumulated neutrophils influence the growth and differentiation of keratinocytes but also activate T cells. These T cells in turn influence the transepidermal neutrophil trafficking through the effect of their lymphokines on the keratinocyte production of proinflammatory mediators. However, no in vivo research has been performed so far that evaluates this hypothesis, and the significance of PMN accumulations in the epidermis still remains unclear. RCM could be a useful tool to investigate these ideas.

In dynamic diseases like psoriasis, conventional histology does not always display characteristic histological features. A biopsy might be obtained too early or too late in the disease process and therefore is not always conclusive. Furthermore, the diagnosis is mainly made clinically and a biopsy is not necessary to render the diagnoses. However, studying dynamic processes like neutrophil accumulation could improve our understanding on the pathogenesis of psoriasis. This might contribute to the development of new or personalized treatments. RCM will be very suitable for the evaluation of dynamic processes and new treatments due to the possibility to obtain multiple in vivo images from the same location non-invasively and over time. A combination of RCM with novel computational methods will further improve the standardization and reliability of this technique.

In summary, this study is the first to establish the dynamics and time phasing of neutrophil migration in vivo in patients with psoriasis. Therefore, RCM could be used to study cyclic neutrophil exocytosis in psoriasis and in other inflammatory skin disorders non-invasively. In future, RCM might distinguish between active or chronic psoriatic lesions, which may contribute to new insights into the pathogenesis of psoriasis.
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References

13. Qazi BS, Tang K, Qazi A. Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis. International journal of inflammation 2011;2011;98468.

34. Talemati H, Tagami H. Quantiﬁcation of chemotactic peptides (IL-alpha; IL-8) and H-8 in psoriatic lesional skin. Archives of dermatology 1991;129;74-80.
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Application of leukotriene B4 and reflectance confocal microscopy as a non-invasive in vivo model to study the dynamics of skin inflammation
Abstract

Background: Application of leukotriene B4 (LTB4) is an established in vivo model that locally induces skin inflammation. Currently in this model, a biopsy is inevitable. In vivo reflectance confocal microscopy (RCM), a non-invasive imaging technique, could overcome this limitation. To find out to what extent RCM may be an in vivo investigative and diagnostic tool in neutrophilic conditions, we studied the dynamics of polymorphonuclear neutrophil (PMN) migration from dermis to stratum corneum using an established LTB4 model.

Methods: Leukotriene B4 was topically applied on the skin of the lower back of seven volunteers. The skin sites were evaluated by RCM for three consecutive days with a 24 h time interval. For histological correlation, 3-mm punch biopsies were obtained. The tissue sections were hematoxylin-eosin and immunohistochemical stained. Minimal and average epidermal thickness was measured.

Results: RCM imaging showed highly reflective ill-defined particles with a granular content throughout the epidermis 24 h after application of LTB4. Over time, the appearance of these cells changed throughout the epidermis. Epidermal thickness increased over time, and the measurements based on the RCM images corresponded very well with the histological images.

Conclusions: RCM was able to visualize PMN migration, accumulation, and degeneration over time in the used LTB4 model. The non-invasive character and the possibility to obtain multiple in vivo images from the same location over time make that RCM in combination with this model a useful tool to study the dynamics and function of PMN in inflammatory processes in the skin.

Introduction

Experiments on inflammatory skin lesions provide us with static and complex information of the disease and inflammatory processes. These in vivo models are rarely used due to their invasive character, induction of inflammation, and the complexity to interpret the data. Information distracted from static data is not suitable to study dynamic in vivo processes like migration, accumulation, and degeneration of polymorphonuclear neutrophils (PMN). Further in diseased skin, it is hard to obtain a biopsy at the most appropriate time, a biopsy can be obtained too early or too late. In vitro studies can be investigated continuously and provide a quite dynamic picture, but lack the complex in vivo environment.

Topical application of human leukotriene B4 (LTB4) is an established in vivo model that locally induces skin inflammation. Leukotrienes are intracellular signaling molecules that are overproduced during an allergic and inflammatory response in several tissues, including the skin.1-3 These leukotrienes are primarily made by inflammatory cells like neutrophils, basophils, eosinophils, macrophages, and mast cells.2-3 Leukotrienes, are metabolites of arachidonic acid derived from the 5-lipoxygenase pathway.1-3 LTB4 has been shown to have potent chemo-attractant activity for PMN, resulting in PMN infiltration into the skin within 24 h after topical application, which subsequently, is followed by a mononuclear infiltrate in the dermis between 48 and 72 h.1-3, 5-7 Topical application of LTB4 causes a dose dependent acute response and attracts a homogeneous population of inflammatory cells. Therefore, this model could be used for studying the specific role of PMN in inflammatory skin diseases like psoriasis.8, 9 Currently, in this model, a biopsy is inevitable. Performing a biopsy has several disadvantages; it is invasive, which in most cases will result in scar formation, and which makes it impossible to evaluate exactly the same location over time. Moreover, biopsies will induce an inflammatory response, which may interfere with the study observations. In the LTB4 model, several biopsies of healthy volunteers are needed to study the inflammatory processes over time. Non-invasive reflectance confocal microscopy (RCM) could be used to overcome these limitations associated with biopsies.

Non-invasive RCM is an in vivo imaging technique, which is able to obtain information on tissue and cell morphology. Imaging the skin is possible up to a depth of 250 µm and the resolution of this technique is comparable to conventional light microscopy.10 Several studies have shown that RCM can be used to diagnose inflammatory skin diseases.11-16 Also, it is a useful device in studying efficacy and side effects of inflammatory skin disease therapies like UVB phototherapy and topical application of corticosteroids and vitamin D3 analogs.17, 18

To date, mainly “lymphocyte-rich” inflammatory skin lesions have been studied by RCM. It is known that inflammatory cells have a relative high refractive index and appear as small homogenous bright particles in RCM images.19 In a case report of a patient with
foliculitis, Gonzalez et al. described bright appearing inflammatory cells in the corneal layer and around hair follicles, corresponding to neutrophils on conventional histopathology. These images were obtained with an older RCM system and are therefore hard to compare with newer images that have a better resolution. With the newer RCM system, only one study reported the appearance of neutrophils within the epidermis. They appear as a shiny granular sludge or polylobulated ill-defined particles with a bright, highly reflective granular content. These cells were morphologically clearly different from lymphocytes.

Polymorphonuclear leukocytes, including neutrophils, have a crucial role in a number of skin diseases including the frequent occurring epidermal dermatoses psoriasis, seborrheic dermatitis, and a number of less frequent skin conditions, which often provide a diagnostic challenge. In psoriatic lesions, it is known that the accumulation of PMN is a dynamic cyclic pattern. Wolberink et al. described that RCM can visualize this dynamics and time-phasing of PMN migration in vivo in psoriatic lesions. Although visualized, the exact mechanism for PMN diapedesis and migration in vivo are not yet fully understood. The findings reported by Wolberink et al. are based on data obtained in a complex diseased environment, which make it hard to unravel the mechanism and dynamics of one specific cell type like PMN. Therefore, to find out to what extent RCM may provide an in vivo investigative and diagnostic tool in neutrophilic conditions, we studied the dynamics of PMN migration from dermis to stratum corneum using the well-established LTB4 model. In particular, we will address the following questions: (i) Is RCM able to visualize PMN migration from dermis to stratum corneum in this model? (ii) Does RCM enable to visualize PMN accumulation and degeneration in the stratum corneum? (iii) Is it possible to differentiate between PMN and mononuclear cells with RCM? Studying the dynamics of PMN migration, accumulation, and degeneration in this PMN-specific skin inflammation model can improve our understanding on the pathogenesis of neutrophilic conditions and therefore might contribute to the development of new or personalized treatments.

Materials and methods

Healthy volunteers

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the local medical ethics committee. Seven healthy adults, one male, and six females, with a mean age of 23 (range 20–28) were included in this study after giving written informed consent. Volunteers with a history of skin disease, an activated immune system, or immunocompromised volunteers were excluded from participation. Measurements were performed at the department of Dermatology, Radboud University Medical Center, Nijmegen, The Netherlands.

LTB4 model

In all volunteers, 10 µl ethanol-LTB4 solution containing 100 ng LTB4 was applied in an 8 mm cylinder at three sites on the lower back skin. The ethanol was evaporated with a stream of nitrogen, resulting in only LTB4 on the skin. Afterward, the test sides were covered with patch test chambers for 8 h to prevent the LTB4 for being displaced.

Reflectance confocal microscopic imaging

RCM imaging was performed with the commercially available VivaScope 1500 system (Lucid Inc., Rochester, NY, USA). This system produces en face images of the skin and is able to examine the skin at a depth of 250 µm. Images were obtained and analyzed using Vivascan 7.0 software (Lucid Inc., Rochester, NY, USA). A more detailed description of the system has been published previously. Wolberink et al. that B lymphocytes did not invade the dermis and epidermis after topical LTB4 application. Therefore only CD3 and elastase staining was performed to stain T lymphocytes and PMN, respectively. The sections for IHC staining were pretreated with peroxidase block (Dako). For the CD3 immunostaining, the sections were antigen retrieved by boiling the sections in EDTA (pH 8.0, 0.5% Tween) for 10 min. Antigen retrieval was not needed to perform the elastase staining. All sections were air dried and immersed in phosphate buffered saline (PBS). This initial step was followed by incubation for 15 min
with 1% bovine serum albumin (Organon Technika, Boxtel, the Netherlands) in PBS. Next, overnight incubation with the primary antibody was performed. This step was followed by incubation with HRP antimouse Envision (Dako) for 30 min. To detect CD3 or elastase, the sections were washed in PBS and the HRP was visualized using 3,3′-diaminobenzidine. Sections were counter-stained with Mayer’s hematoxylin (Sigma, St Louis, MO, USA) and were after dehydration mounted in Permount (BDH Chemical, Poole, UK). Finally, the sections were photographed using a microscope (Axioskop2 MOT; Zeiss, Jena, Germany), digital camera (Axiocam MRC5; Zeiss), and AxioVision software (Zeiss).

Analyses
All RCM images were evaluated for the presence of highly reflective inflammatory cells. The location and the appearance of these cells were described. For each time point, mean values ± SD for average thickness of the stratum spinosum were calculated based on the histological images (epidermal surface/length). The minimal epidermal thickness was determined for the RCM as well as the histological images. For the RCM images, two Vivastacks were evaluated. The stratum spinosum thickness was determined by counting the steps (4.5 µm) in depth, starting at the stratum granulosum till the first dermal papillae appeared. The minimal epidermal thickness present in the histological sections was determined by the average of the five thinnest points of the epidermis. RCM and histological minimal epidermal thickness values were compared and tested for statistical differences by an independent t-test analysis. Throughout the analysis, p values <0.05 were considered significant.

Results
PMN appearance through the stratum spinosum
Twenty four hours after topical application of LTB4, in six of the seven volunteers, highly reflective ill-defined particles with a granular content in the stratum granulosum and spinosum were observed by RCM (Figure 1). The clinical, histological, and RCM data of one volunteer showed the absence of an inflammatory response. For the volunteers that showed a response, the highly reflective cells in the stratum spinosum corresponded to the elastase positive cells observed in the elastase stained sections and therefore were considered to be PMN. CD3 positive cells were not present at this time point (Figure 2). With RCM, the PMN appeared differently from bottom to top of the epidermis, representing the changes of these cells during migration. At the deep stratum spinosum, closely to the dermal-epidermal junction, these cells appeared as solitary small round to oval bright particles. Moving more upward, these cells transformed into larger ill-defined granulated highly reflective cells. The massive infiltrate of highly reflective PMN cells disturbs the regular honeycomb pattern, which is normally seen by RCM at the level of the stratum

Figure 1 Application of leukotriene B4 (LTB4) causes influx of highly reflective cells in the epidermis. Reflectance confocal microscopy Vivastack images of normal skin before LTB4 application (a–d) and 24 h after LTB4 application (e–h). The stratum granulosum (a) and stratum spinosum (b), showing a regular honeycomb pattern.
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spinosum (Figure 1). PMN visualization in the dermis was not possible due to limited resolution in depth. The resolution was limited by the highly reflective PMN cells in the upper epidermal layers and the development of mild acanthosis (Figure 3).

Accumulation and degeneration of polymorphonuclear neutrophils over time

The accumulations of highly reflective PMN were seen at the upper stratum spinosum and the stratum corneum. The formation of these accumulations started 24 h after LTB4 application. At this time point large, highly reflective individual cells and some clusters of cells appeared. Interestingly, high in the stratum spinosum, these cells seem to vibrate to some extent. This is demonstrated in the video, which is available at http://onlinelibrary.wiley.com/doi/10.1111/srt.12181/suppinfo. Forty eight hours after topical application of LTB4, the appearance of these cells became less reflective and more homogenous with vague cellular contours. One day later, some rests of the previously highly reflective cells were present. These RCM features indicated degeneration of the PMN accumulations, which was confirmed by the CD3 and elastase IHC staining. CD3 positive cells were only visible in the dermis at 48-72 h after application of LTB4. These cells were not detectable with RCM due to the location in the dermis and the thickening of the epidermis (Figure 2 and 3).

Stratum spinosum thickness

Based on the histological sections, topical application of LTB4 resulted in an increase in the mean stratum spinosum thickness over time (Figure 3a). Twenty four and 48h after LTB4 application, the maximum stratum spinosum depth could not be determined with RCM due to the highly reflective clusters of cells located high in the stratum spinosum. These cells reflect a large amount of the laser light and therefore prevent further penetration of the light into the dermis. Therefore, the mean stratum spinosum thickness could not be determined by RCM. However, it was possible to determine the minimum stratum spinosum depth, defined as the depth at which the first dermal papillae were visible. In the studied skin inflammation model, this minimal epidermal thickness increased over time and measurements based on the RCM images corresponded very well with measurements performed on the histological images. No significant difference was found between the two methods (Figure 3b).
Discussion

In this study, the ability and value of non-invasive in vivo RCM imaging as a tool for studying dynamic inflammatory processes in the skin was evaluated by using the LTB4 skin inflammation model. The changes in the skin were followed with RCM during the first 72 h after topical application of LTB4. RCM images were correlated with conventional histopathology.

Conventional histopathological HE stained sections displayed that topical application of LTB4 resulted in PMN infiltration in the epidermis between 8 and 48 h. Our findings are in line with results reported by others. RCM was able to visualize the highly reflective PMN over this time interval. PMN migration and cellular appearance was imaged from the dermal-epidermal junction up to the stratum corneum. High in the stratum spinosum, PMN appearance seen with RCM fitted well to the description of neutrophils by Debarbieux et al. Over time, the dynamic process of PMN accumulation and degeneration at the level of the stratum corneum was clearly visible with RCM. The observed morphological changes correspond to the cell biological knowledge of PMN migration, accumulation, and degeneration, which is also described in psoriatic lesions. Individual cells and clusters of PMN appeared as highly reflective cells, whereas degenerated cell fragments appeared as dense low reflective structures in which no cellular contours could be identified, likely to correspond to the pyknosis and karyorrhexis stages of apoptotic cells.

Epidermal thickness as a result of LTB4 application is a known phenomenon. However, to the best of our knowledge, we are the first who demonstrated the accurate correlation between stratum spinosum thickness measurements on histological sections and RCM images. The results of this study confirm that RCM can be used to non-invasively evaluate dynamic inflammatory processes and cell morphology in the epidermis.

Debarbieux et al. stated that with RCM, neutrophils appear morphologically clearly different from lymphocytes. However, it was not investigated whether it is possible to differentiate between these two cell types within one lesion. We tried to study this in the LTB4 model in which the PMN infiltrate is followed by a mononuclear cell infiltrate in the dermis 48-72 h after application. Unfortunately, the thickening of the epidermis, as a result of LTB4 application, in combination with relative high reflectivity of inflammatory cells in the epidermis limits light penetration into the dermis. Therefore, it was not possible to distinguish between PMN and mononuclear cells in this model using RCM imaging. However, we would like to stress that the use of RCM in combination with the LTB4 model is an excellent method to study PMN dynamics and morphology in vivo.

The vibration of the PMN highly in the epidermis is a remarkable new feature, which seem to be very specific for PMN. Probably, this vibration on the real-time images is caused by the complexity of these polynucleated cells. The light reflected by these complex and large cells will be more scattered compared to cells with a small round nucleus. Further, it might be possible that these cells really vibrate to some extent, as they are trafficking...
toward the stratum corneum. However, the velocity of the trafficking of these cells is not yet investigated.

In summary, RCM seems to be a promising technique in investigating and diagnosing neutrophilic skin conditions, which often provide a diagnostic challenge. In these cases, obtaining a good and efficient diagnosis will result in direct proper treatment and might even prevent treatment failure. The non-invasive character, the specific PMN infiltrate, and the possibility to obtain multiple in vivo images from the same location over time make that RCM in combination with the LTb4 model a useful tool to study the dynamics and function of PMN in inflammatory processes in the skin. Studying PMN migration, accumulation, and degeneration in this skin inflammation model can improve our understanding on the pathogenesis of neutrophilic conditions and might contribute to the development of new therapies.

References

16. Debarbieux S, Depaepe L, Pouillahon N, Dalle S, Balme B, Thomas L. Reflectance confocal microscopy charac-
teristics of eight cases of pustular eruptions and histopathological correlations. Skin research and technology: official journal of International Society for Bioengineering and the Skin 2013;19:e444-e452.


4
Skin damage
4.1

The potential of the skin as a readout system to test artificial turf systems: clinical and immunohistological effects of a sliding on natural grass and artificial turf.
Abstract

The purpose of this study was to investigate the interaction of skin with natural grass and artificial turf at clinical, histological and immunohistochemical level. Therefore, 14 male volunteers performed slidings on dry natural grass, wet natural grass and artificial turf. Directly and 24 h after the sliding, a clinical picture and a 3-mm punch biopsy of the lesion were taken. Paraffin sections were hematoxylin-eosin stained. Immunohistochemistry was performed for CD3, hBD-2, K16, K10, Ki67 and HSP70. Clinically, a sliding performed on artificial turf caused less erythema but more abrasion compared to natural grass. At histological level, artificial turf or dry natural grass damaged the stratum corneum the most. Directly after the sliding, CD3, hBD-2, K16, K10, Ki67 and HSP70 expression was normal. 24 h after a sliding on artificial turf or dry natural grass, an increase of K16, hBD-2 and HSP70 expression was observed. In this pilot study, it was not possible to clearly distinguish between skin damage induced by a sliding on artificial turf and natural grass. However, small differences at clinical and histological level seem to exist. This demonstrates the potential of the skin as readout system to evaluate artificial turf systems and mechanical skin damage.

Introduction

Soccer, also known as football, is the most popular sport worldwide with approximately 200 million players. Like other popular team sports such as ice hockey, handball, basketball and rugby, soccer is a sport with a relatively high risk of injury. Participation in this sport is still increasing, leading to an increasing frequency of injuries resulting in higher costs for treatment. The incidence and characteristics of soccer injuries during soccer matches at amateur and top level are well documented. However, skin lesions are rarely described. Severity and frequency are underestimated because an injury is generally defined as any physical complaint caused by soccer that lasted for more than 2 weeks or resulted in absence from a subsequent match or training session. Although missing a match or training due to a skin injury is uncommon, the related discomfort can still negatively influence players' performance.

A sliding is commonly performed during a soccer match and results in redness (erythema) and abrasion of the skin. Such surface-related traumatic damage is usually a minor injury, but it can be serious if it covers a large area or when foreign materials becomes imbedded in the skin lesion. The effects of artificial turf and natural grass on surface-related traumatic injuries in soccer suggests that surfaces with artificial turf produce more abrasion injuries than surfaces with natural grass. However, these descriptive studies are only performed in small groups. Playing on different types of surfaces has been suggested to cause difference in injury pattern and mechanism. Further, alternation between different types of playing surfaces is related to a higher injury risk.

Artificial turf became used widespread for baseball and soccer in the United States and Canada in the 1970s, in both outdoor and indoor stadiums. In Europe, some soccer clubs installed artificial surfaces in the 1980s. During this period, artificial turf gained a bad reputation with fans and especially players, since this artificial turf caused more injuries than natural grass. The use of artificial turf for soccer purposes was banned by FIFA, UEFA and many domestic associations. However, artificial turf systems could be very useful in regions of the world with a climate that makes growth of adequate natural grass difficult. Therefore in the last decade, new artificial playing surfaces have been developed. Some of these surfaces were specifically designed for soccer. The basic construction of the latest generation soccer specific artificial turf is a blend of grass-like fibers attached to a special backing containing a mix of sand and rubber brushes. This construction has proven to be the most favorable for soccer to date. These “next generation” surfaces are often virtually indistinguishable from natural grass when viewed from any distance. Besides, they are generally regarded as being about as safe to play on as natural grass. In Europe, UEFA has approved the use of these artificial pitches at national and club level since the 2005-2006 season. Therefore, it is possible that soccer matches are played on artificial turf in the Champions’ League, UEFA Cup or qualifiers for the World Cup and European championships.
Soccer injuries predominately affect the ankle, knee and muscles of the thigh and calf. The incidence of these injuries on artificial turf compared to natural grass is therefore studied the most. However, to the best of our knowledge no studies have been performed that evaluate skin lesions after a sliding on natural grass and artificial turf. For this reason, we have conducted a descriptive study to evaluate the clinical, histological and immunohistochemical effects of a sliding on different playing surfaces.

**Methods**

**Skin samples**

14 male healthy volunteers from the same soccer team were included in this study after giving written informed consent. All participants were active amateur soccer players. The age of the volunteers ranged from 18 to 25 years, with a mean age of 22 years. Their height varied between 178-192 cm and the weight was between 71-94 kg resulting in a body mass index between 20-25. The skin type of the volunteers was type II or III (white skin). The volunteers were asked to perform a “standard sliding” 1-10 times on 3 different types of playing surfaces. The running distance before a sliding was equal in all volunteers. Further, all volunteers played soccer at the same level and they were equally trained. Slidings on natural grass were carried out in the soccer stadium Amsterdam Arena, the Netherlands. Two grass conditions were tested. A dry playground with the stadium roof closed and the same playground after 3 min water sprinkling (standard procedure in advance of a soccer game). Slidings on artificial turf were performed indoors on a third generation artificial turf system. This was a grass carpet containing fibrillated LSR yarn. The grass piles had a length of 6 cm and as basis 2 cm infill sand (M4C) and on top 2 cm of granulated thermoplastic elastomer. The lesions caused by the sliding were clinically characterized in a subjective way by an experienced not blinded dermatologist directly after the sliding and 24 h later. For this purpose, a clinical picture was taken and a description was given about, the size of the lesion and the degree (mild, moderate, severe) of erythema and abrasion. This study was approved by the local medical ethics committee and was conducted according to the principles of the Declaration of Helsinki. The study meets the ethical standards of the journal.

**Histology and immunohistochemistry**

For histological evaluation by a dermatologist and a biologist, directly after the sliding and 24 h later 3-mm punch biopsies were taken under local anesthesia with 1 % Xylocain/Adrenaline. These biopsies were embedded in paraffin after 4 h fixation in formaldehyde. Paraffin sections (6 µm) were processed side by side and dewaxed with histosafe (Adamas, Rhenen, The Netherlands) followed by rehydration in decreasing concentrations of alcohol (100–50 %) and demineralized water. These sections were hematoxylin-eosin (HE) stained for assessment of histopathological features. Furthermore, immunohistochemical (IHC) staining was performed with monoclonal antibodies specific for CD3 (1:500) clone F7.2.38, human beta defensin-2 (hBD-2) (1:10 000) clone ab9871 (Abcam, Cambridge, UK), cytokeratin 16 (K16) (1:500) clone LLL025 (Monosan, Uden, Netherlands), cytokeratin 10 (K10) (1.5 000) clone RKS560 (Monosan, Uden, Netherlands), K67 (1.50) clone M18-1 (Dako, Copenhagen, Denmark), and heat shock protein 70 (HSP70) (1:50 000) clone W27 (Santa Cruz, Santa Cruz, USA) together with a hematoxylin counterstaining.

The sections for the CD3, K10, K67 and HSP70 staining were pre-treated with peroxidase block (Dako, Copenhagen, Denmark) and the sections for hBD-2 with 20% normal rabbit serum. For the CD3 immunostaining, the sections were antigen retrieved by boiling the sections in EDTA (pH 8.0, 0.5 % Tween) for 10 min. For K16 and K67, pre-treatment with peroxidase block was followed by boiling of the sections in a 10-mM citrate buffer solution (pH 6.0) (high-temperature microwave oven retrieval technique by Cattoretti et al.) at 450 W in a microwave oven for approximately 10 min and left to cool for 45 min. Sections for K10 staining were antigen retrieved by trypsinization with trypsin-CaCl₂ solution for 15 min at 37 °C. Afterwards, all sections were air dried and immersed in phosphate buffered saline (PBS). For all staining except hBD-2, this initial step was followed by incubation for 15 min with 1 % bovine serum albumin (Organon Technika, Boxtel, the Netherlands) in PBS. Next for all staining, overnight incubation with the primary antibody was performed. For CD3, K16, K10, K67 and HSP70, this step was followed by incubation with HRP anti-mouse Envision (Dako, Copenhagen, Denmark) for 30 min. The sections for hBD-2 were incubated with rabbit anti goat IgG biotinylated Vectastain ABC-elite kit (Vector laboratories, Burlingame, USA). To detect CD3, hBD-2, K16, K10, K67 or HSP70, all sections were washed in PBS and visualized using 3,3-diaminobenzidine (DAB). Sections were counter-stained with Mayer’s haematoxylin (Sigma, St Louis, USA) and after dehydration mounted in Permount (BDH chemical, Poole, England). Finally, the sections were photographed using a microscope (Axioskop2 MOT, Zeiss), digital camera (Axivision software (Zeiss).

**Results**

The aim of this study was to investigate the effects on the skin of a sliding, on natural grass or artificial turf. Therefore, the clinical appearance of skin lesions caused by a sliding is described. Directly after the sliding, the size of the skin lesions on the thigh of the volunteers caused by natural grass or artificial turf varied between 5–90 cm² and 20–70 cm², respectively. All playing surfaces caused erythema of the skin, however based on the findings of a dermatologist a sliding on artificial turf resulted in milder erythema compared to dry and wet natural grass. In contrast, almost no abrasion was found after a sliding on natural grass, which did appear after a sliding on artificial turf (Figure 1 and Table 1). Further, wet natural grass caused less erythema and abrasion compared to dry natural grass. Almost all lesions were clinically improved 24 h after the sliding, which was illustrated
by reduction of erythema (Figure 1 and Table 1). Inter-individual differences were observed in all groups. In addition to these observations, 2 aberrant cases were found. One of the volunteers who performed a sliding on artificial turf did not show any features of skin damage. In a second volunteer, an extreme inflammatory reaction accompanied by pinpoint bleedings was observed after a sliding on wet natural grass. Histology confirmed the clinical observations of these 2 cases. Overall, it seems that a sliding on natural grass results in more erythema but less abrasions compared to a sliding on artificial turf.

In addition to the clinical evaluation, HE and IHC stains were performed to study histological changes in the skin lesions after a sliding on the tested playing surfaces. In 13 volunteers, a sliding resulted in total or partial removal of the stratum corneum. However, compared to wet natural grass, artificial turf and dry natural grass disrupted the stratum corneum the most (Figure 2).

Figure 1  Clinical appearance of skin lesions caused by a sliding. Representative clinical pictures of skin lesions on the thigh of the volunteers caused by a sliding on different playing surfaces: a) Lesion as result of a sliding performed on dry natural grass directly (1) after a sliding and 24 h later (2), demonstrating severe erythema and mild abrasion. b) Skin lesion as a result of a sliding on artificial turf directly (1) after the sliding and 24 h later (2), illustrating moderate abrasion and mild erythema. In the middle of both pictures a2 and b2, a small wound with a stitch can be observed at the location where the 3-mm biopsy was taken.

Figure 2  Skin histology after a sliding on different playing surfaces. Representative pictures (20 × magnification) of HE stained skin biopsies after a sliding on 3 different playing surfaces. The columns consecutively represent the histology of the skin lesions directly after a sliding and 24 h later. (a, b) Skin histology after a sliding on artificial turf, illustrating removal of SC (black arrow). (c, d) Shows the skin after a sliding on dry natural grass, which results in removal of the SC. (e, f) Demonstrates skin histology after a sliding on wet natural grass, showing less disruption of the SC compared to dry natural grass and artificial turf. (g) Normal skin histology.
Discussion

This study showed that there is no evidence of more skin related traumatic injuries when a sliding was performed on artificial turf compared to natural grass. This is in agreement with a study performed by Ekstrand et al. who investigated the risk of injury in elite soccer played on artificial turf vs. natural grass. Both studies were carried out on improved third generation artificial turf, which is especially designed for soccer and has playing characteristics similar to natural grass. These artificial turf pitches were introduced in the late 1990s and are officially allowed by FIFA and UEFA for international matches.11

Aberrant expression of CD3, hBD-2, K16, K10, Ki67 and HSP70 was not found directly after the sliding. Interestingly 24 h later, an increased epidermal K16 expression was observed as result of a sliding on artificial turf or dry natural grass (Figures 3a–b). This K16 induction was not clearly accompanied by K10 reduction, which indicates mild disturbed epidermal differentiation. In addition to changes in keratinocyte differentiation, a sliding induced epidermal thermal stress response and a component of the skin barrier function was studied by HSP70 and hBD-2, respectively. 24 h after a sliding on artificial turf and dry natural grass, suprabasal expression of HSP70 and hBD-2 was observed in 11 out of 12 volunteers, especially at areas with an undisrupted stratum corneum (Figures 3d, e, g, h). This increase of K16, hBD-2 and HSP70 expression within 24 h was not found in volunteers who performed a sliding on wet natural grass, indicating a normal epidermal differentiation, no thermal stress induction and a normal skin barrier function (Figures 3c, f, i). These observations correspond to the results of the HE staining. Further for all playing surfaces, infiltration of inflammatory cells and keratinocyte proliferation did not significantly increase 24 h after a sliding.
We observed small differences between sliding damaged skin caused by wet natural grass, dry natural grass and artificial turf at clinical and histological level. This is in contrast to the absence of a difference observed in the number of traumatic injuries. Based on our clinical findings, we confirm the suggestion by others that a sliding on natural grass results in more erythema but less abrasions compared to a sliding on artificial turf.\textsuperscript{7, 8, 9} At histological level, a sliding on artificial turf and dry natural grass reduced the skin barrier function within 24 h after the sliding. This was depicted by increased hBD-2 expression, which is known for its antimicrobial activity and can therefore be related to reduced skin barrier function.\textsuperscript{10, 11} In addition, this reduced skin barrier function is accompanied by thermal stress induction that is represented by HSP70. In inflammatory skin diseases with reduced skin barrier function, like psoriasis and atopic dermatitis, HSP70 is also involved.\textsuperscript{12, 13} In contrast, water sprinkling of natural grass seems to prevent this sliding induced thermal stress induction and skin damage. Therefore water sprinkling of natural grass before a soccer match seems to be a good approach in reducing soccer match related skin lesions and improvement of players comfort. This finding makes it interesting for future studies to evaluate the effect of water sprinkling of artificial turf on the incidence and severity of soccer match related skin lesions. Further, in a larger study implementation of a tool to measure the amount of erythema and abrasion, for example Courage and Khazaka, could be beneficial. Wound, burns and friction injuries that effect skin comfort are more common in severe injury it still causes players discomfort. The impact of sliding related injuries on players discomfort may therefore be underestimated. So the observed differences may be important in studying users skin comfort. However, differences in sliding techniques or pain perception can influence the results.

Previous studies only investigated severe soccer related injuries on natural grass versus artificial turf. They did not investigate skin damage and its influence on players comfort on these surfaces. We showed that skin damage can be evaluated at both clinical and biological level. This illustrates that besides tribology, biology is of importance in the development of artificial turf systems and testing their users comfort. This invasive study therefore shows the potential of the skin as a read-out system for skin damage and contributes to the development of novel non-invasive in vitro imaging methods to study skin damage and skin-material interaction.

References
4.2

Combining tape stripping and non-invasive reflectance confocal microscopy: an in vivo model to study skin damage
Abstract

Background: Evaluation of (immuno)-histological and cell biological changes in damaged skin requires often an invasive skin biopsy, making in vivo models inappropriate to study skin damage. Reflectance confocal microscopy (RCM) might overcome this limitation. Therefore, we evaluated the use of a tape-stripping model in combination with RCM to provide morphological data on skin damage and recovery.

Methods: In 25 volunteers a tape-stripping stimulus was applied. The skin was imaged with RCM during 1 week and 3-mm punch biopsies were obtained.

Results and conclusion: Strong correlations between epidermal thickness determined by RCM and conventional histological measurements were found. RCM thickness measurements correlated well with epidermal proliferation. The 10x or 15x repeated tape stripping resulted in skin damage similar to acute stripping. Mild repeated tape stripping showed no skin damage. Overall, we demonstrated that non-invasive RCM in combination with tape stripping could be used as model to obtain morphological and cell biological data on skin-material interactions.

Introduction

Skin serves as the interface between humans and their environment, thereby protecting the body against negative environmental influences. This is achieved by its multilayered structure. The barrier function is mainly regulated by the outermost layer of the skin, called the stratum corneum (SC). This layer is produced by the process of cornification, resulting in layers of tightly packed anucleated skin cells mainly full of keratin. These cells are embedded in a lipidic intercellular matrix composed of ceramides, long-chain free fatty acids and cholesterol. In addition to the SC, the antimicrobial and innate immunity barrier is regulated by the living epidermal layer below the SC.

Disruption of the skin barrier function is often studied at a biological level in skin diseases e.g. in wounds. However, studies on mechanical skin damage are rare. Mechanical skin injury can be caused by performing sports, in case the skin interacts with the playground, or in a car accident were airbags protect against life threatening injuries but meanwhile cause abrasion type of skin injuries. The cell biological effects on the skin after skin-material interaction however, may be of importance when developing skin friendlier materials and products. With (immuno)-histopathology these cell biological changes in the skin can be studied. At present, for this purpose an invasive skin biopsy is still required, which is troublesome while studying skin damage as result of skin-material interaction, as the extra inflammation caused by the biopsy will interfere with the results of the experiment.

Currently available methods to investigate skin-material interactions do not use the skin as readout system. These models only study the interaction between two different kind of materials, one of which represents the skin. It is difficult to translate results obtained by these methods to human skin, since clinical appearance and skin histology cannot be evaluated over time. An in vivo skin damage model combined with a non-invasive readout technique could build a bridge between the clinic, fundamental science and industry, contributing to the development of user-friendlier materials and products.

The in vivo sellotape-stripping model, which is commonly used for studying the skin barrier function, epidermal growth and concurrent immune responses, might be useful to study skin damage related to skin-material interaction. This model is a minimal invasive procedure for the removal and sampling of the SC. In vivo biotribology measurements have been performed in this model, however a skin biopsy is still required to evaluate the histological and cell biological changes in the skin. While studying skin damage, this is troublesome as mentioned above, as the extra inflammation caused by the biopsy will interfere with the experiment results.

Non-invasive imaging tools such as high-frequency ultrasound and optical coherent tomography have resolutions that only reveal architectural changes of the skin. These techniques do not allow identification of cellular or sub-cellular structures and therefore
will not yield the missing information in the field of mechanical skin damage. Reflectance confocal microscopy (RCM) on the other hand is a non-invasive imaging technique, which is used for in vivo evaluation of skin morphology, that offers a resolution and contrast comparable to conventional light microscopy.\textsuperscript{11, 18} This technique allows to image cells to a depth of about 250 µm. In confocal images, contrast is provided by refractive index differences between cells and surrounding tissue. The contrast of in vivo RCM-imaging of the skin is mainly provided by melanin and keratin. In contrast to these highly reflective structures, white blood cells, chromatin, collagen, and elastin show lower reflection.\textsuperscript{19-21}

This imaging technique is currently used for diagnosis of skin cancer and inflammatory skin diseases.\textsuperscript{22-28} Further, it is shown that RCM can be used for follow-up of therapy and studying dynamic inflammatory processes in vivo.\textsuperscript{24-25}

The aim of the present study is to evaluate the tape-stripping model in combination with non-invasive in vivo RCM in order to study mechanical skin damage and recovery over time. In the standard tape-stripping model the whole SC is stripped off, thereby significantly disturbing the skin barrier function. However, after skin-material interaction, total removal of the corneal layer is not always the case.\textsuperscript{1} Gerritsen et al. studied single versus constituted tape-stripping, to evaluate the immune response.\textsuperscript{11} In line with this, we questioned whether the tape-stripping model can be used as an acute (e.g. sliding on a playing ground or airbag impact) as well as a chronic (e.g. daily shaving or repeated skin scrubbing) mechanical skin damage model. Combining the tape-stripping model with RCM imaging may prevent the necessity to perform skin biopsies in healthy volunteers and may provide a non-invasive skin damage model in which the skin is used as a readout system. In this model it is possible to monitor skin changes over time. Therefore, this non-invasive model may increase our knowledge about mechanical disruption and recovery of the skin at a (cell)-biological level over time, thereby contributing to the development of more skin friendlier material and products.

### Materials and Methods

#### Healthy volunteers

In this study, 25 healthy adults, 9 males and 16 females, with a mean age of 24 years (SD 4.9) and skin type I, II or III were included after giving written informed consent. Volunteers with a history or signs of chronic skin diseases, disturbed wound healing, an activated immune system, or immunocompromised volunteers were excluded from participation. Two weeks prior to the experiments, volunteers were not allowed to expose their lower back skin to sunlight. This study was conducted according to the principles of the Declaration of Helsinki and was approved by the local medical ethics committee. Measurements were performed at the department of Dermatology, Radboud University Medical Center, Nijmegen, The Netherlands.

### Tape-stripping procedure

The volunteers were randomized over two groups, one received an acute tape-stripping stimulus and the other group received a repeated tape-stripping stimulus. In all volunteers, tape-stripping was performed on several sites (2x1 cm) on the lower back skin. For the acute skin damage stimulus (n=12), the adhesive tape (Sellotape Original 1109, Borehamwood, UK) was sequentially applied and removed (25-30x) onto the skin surface until the skin glistened. In this way, all layers of the SC were removed and provided a standardized trauma of the skin.\textsuperscript{13, 20} For the repeated skin damage stimulus (n=13), three different frequencies of tape-stripping were used for 5 consecutive days. The frequencies included 5x (n=4), 10x (n=4) or 15x (n=5) tape-stripping, respectively. All time intervals were based on a pilot study performed at our department and a literature search.\textsuperscript{11, 26, 27}

#### RCM imaging

RCM imaging was performed with the commercially available VivaScope 1500 system (Lucid Inc, Rochester, NY, USA). Images were obtained and analyzed using Vivascan 7.0 software (Lucid Inc., Rochester, NY, USA). A more extensive description of the RCM technology has been published previously.\textsuperscript{17-18, 21}

RCM images and clinical pictures with the Vivacam (Lucid Inc., Rochester, NY, USA) were obtained before and instantly after the first tape-stripping moment. These measurements were repeated at 24 h, 72 h and 1 week after the first measurements. At 48 h and 96 h in combination with the tape-stripping, additional measurements were performed in the repeated tape-stripping group. The RCM images were obtained according to a standardized protocol. A horizontal map of 4 x 4mm (Vivablock) was made at the level of the SC, stratum spinosum, dermal-epidermal junction and papillary dermis. Within the area of interest, two vertical mappings (Vivastack) were performed. This mapping included a series of images of 0.5 x 0.5 mm in depth with steps of 4.5 µm, started at the top of the SC until the papillary dermis. Moving features were captured by short videos.

#### Histology and immunohistochemistry

To correlate the RCM images, 3-mm skin biopsies were taken under local anaesthesia with 1% Xylocain/Adrenalin. A biopsy of healthy skin was obtained as reference and internal control. Taking the ethical aspects into account, a biopsy was not obtained at 48 h in order to minimize the number of biopsies. The biopsies were embedded in paraffin after 4 h fixation in formalin. Paraffin sections (6 µm) were deparaffinized with histosafe (Adamas, Rhenen, The Netherlands) followed by rehydration in decreasing concentrations of alcohol (100-50%). The sections were hematoxylin-eosin (HE) stained for assessment of histo-pathological features. In addition, immunohistochemical (IHC) stainings were performed with monoclonal anti-human primary antibodies specific for CD3 (1:500, clone F7.2.38, Abcam, Cambridge, UK), elastase (1:5000, clone NPS7, Dako, Copenhagen, Denmark), CD31(1:100, clone JC70A, Dako, Copenhagen, Denmark), cytokertatin 16 (K16) (1:500, clone
were pretreated with 3% H₂O₂ in methanol for 15 minutes, in order to omit endogenous differentiation and proliferation. The sections for the CD3, elastase, CD31 and Ki67 staining blood vessels. Cytokeratin 16 and Ki67 were performed in order to evaluate keratinocyte morphonuclear leukocytes (PMN), respectively. CD31 stains the endothelial cells of the Denmark). CD3 and elastase stainings were performed to stain T-lymphocytes and poly-LL025, Monosan, Uden, The Netherlands) and Ki67 (1:100, clone MIB-1, Dako, Copenhagen, Copenhagen, Denmark). CD3 and elastase stainings were performed to stain T-lymphocytes and poly-

The thickness of the living epidermis significantly increased 72 h after acute tape-stripping stimulus, significant differences in SC thickness were found between the acute and repeated (5x) tape-stripping model. At this time point, in the acute model, the SC was totally removed whereas in the 5x repeated tape-stripping group a minimal decrease in thickness was observed (Figure 2a). Because of large standard deviations we did not observe significant differences between the other stimuli (Figure 2c). However, instantly after the first tape-stripping, 10x and 15x tape-stripping seem to result in less skin damage compared to the acute stimulus. In contrast to acute tape-stripping, independent of the tape-strip frequency, the total SC was never totally removed during and after repeated tape-stripping (Figures 2a and c).

**Inflammation and epidermal proliferation**

CD3 positive cells or elastase positive cells were scored in the IHC stained sections. The scoring was defined as normal (n), mild increased influx of inflammatory cells (+/-), severe increased influx of inflammatory cells (+). In addition, all RCM images were evaluated for the presence or absence of highly reflective inflammatory cells in the epidermis and dermis. In the Ki67 stained tissue sections, the number of Ki67 positive nuclei per mm epidermis were counted using AxiosVision software 4.8 (Zeiss). In addition, cytokeratin 16 expression was scored as absent (-), mild expression (+/-) or strong expression (+). All HE stained sections and all RCM images were scored for the presence or absence of parakeratosis and spongiosis. Further, loss of the honeycomb pattern in the epidermis was evaluated.

**Vascularisation, ratio basal membrane-stratum corneum and number of papillae**

In the dermis, the area positive for CD31 IHC staining was measured using ImageJ software 1.46. Further the ratio between the length of the basal membrane and length of the SC was determined. With RCM the number of dermal papillae per mm² was counted at the level of the dermal epidermal junction.

**Statistical analysis**

Correlations between RCM, histology and IHC measurements were calculated by a Pearson correlation analysis, using GraphPad Prism software 5.03. ANOVA analysis with post-hoc testing was used to compare values of the different groups and time points. Throughout the analysis, p-values <0.05 were considered significant.

**Results**

**Correlation between RCM and histology thickness measurements**

A strong correlation (Pearson r = 0.88) was found between histology and RCM images for both the SC and living epidermal thickness measurements (Figure 1).

As expected, instantly after the tape-stripping stimulus, significant differences in SC thickness were found between the acute and repeated (5x) tape-stripping model. At this time point, in the acute model, the SC was totally removed whereas in the 5x repeated tape-stripping group a minimal decrease in thickness was observed (Figure 2a). Because of large standard deviations we did not observe significant differences between the other stimuli (Figure 2c). However, instantly after the first tape-stripping, 10x and 15x tape-stripping seem to result in less skin damage compared to the acute stimulus. In contrast to acute tape-stripping, independent of the tape-strip frequency, the total SC was never totally removed during and after repeated tape-stripping (Figures 2a and c). Acute tape-stripping resulted in a significant increase of SC thickness 72 h after tape-stripping and increased even more a week after the stimulus was applied (Figure 2c).

After the last tape-stripping moment in the 10x and 15x tape repeated stripping groups, SC thickening was comparable to acute tape-stripping. This SC thickening was never observed after 5x tape-stripping for 5 consecutive days (Figures 2a and c).

The thickness of the living epidermis significantly increased 72 h after acute tape-stripping (Figures 2b and d). Although as late as one week after the tape-stripping was started, this thickening was also observed in the 10x and 15x repeated tape-stripping.
groups. For the 5x repeated tape-stripping, again no significant differences were found between measurements; the thickness of the living epidermis remained equal (Figures 2b and d).

**Figure 1** Correlation between thickness measurements based on histological sections and RCM imaging. a) Stratum corneum thickness measurements. b) Thickness of the living epidermis.

**RCM thickness measurements as a measure for keratinocyte proliferation in vivo**

In the basal layer, a significantly increased number of proliferating keratinocytes was present 72 h after acute tape-stripping. Although the number of Ki67 positive cells decreased 96 h and 1 week after tape-stripping, it remained significantly increased compared to the number of Ki67 positive keratinocytes before, instantly after and 24 h after tape-stripping (Figures 3a, c, and d). The 10x and 15x repeated tape-stripping stimulus followed this line with a small delay in time, showing a peak in proliferating keratinocytes 96 h after the first tape-stripping stimulus. The number of Ki67 positive cells in these groups was lower compared to acute tape-stripping. One week after the first stimulus, the number of proliferating cells decreased as well in these two repeated tape-stripping groups. In line with the thickness measurements, 5x repeated tape-stripping showed a constant number of Ki67 positive cells, with no significant differences between time points (Figure 3c). A Pearson analysis resulted in a moderate to strong correlation (r=0.54) between the number of Ki67 positive keratinocytes and the epidermal thickness (SC + living epidermis) (Figure 3b). For all stimuli, cytokeratin 16 expression showed a trend comparable to the Ki67 expression (Table 1, Supplementary Figure).
Figure 2. Continued. d) Graphs of the average stratum corneum thickness for each tape-stripping stimulus, including standard deviations.

d) The average thickness with standard deviation of the living epidermis thickness measurements for each tape-stripping stimulus. Significant differences between measurements are indicated by an asterisk (*).

Table 1 Overview of the scoring of cytokeratin 16, CD3 and elastase immunohistochemical staining in all tape-stripping stimuli over time. Cytokeratin 16 expression in the epidermis was scored as absent (-), mild expression (+/-), strong expression (+). CD3 and elastase positive cells were not present in the epidermis, therefore they were only scored in the dermis. The presence of these inflammatory cells was scored as normal (N), mild increased dermal infiltrate (+/-), strong increased dermal infiltrate (+).

<table>
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<tr>
<th>Time</th>
<th>Cytokeratin 16</th>
<th>CD3</th>
<th>Elastase</th>
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Figure 3  Epidermal proliferation as result of acute and repeated skin damage. **a)** Overview of Ki67 expression in all tape-stripping groups over time. **b)** Correlation between epidermal thickness and the number of Ki67+ nuclei per mm epidermis.

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Figure 3  Continued. **c)** Graphs of Ki67 expression, including standard deviation, in each tape-stripping group. Significant differences ($p<0.05$) are depicted with a asterisk (*). **d)** Representative photographs of Ki67 immunohistochemically stained tissue sections before and after acute skin damage.
Parakeratosis, epidermal atypia and spongiosis
As a result of hyperproliferation and wound recovery, parakeratosis was observed 24 h, 72 h and 1 week after acute skin damage. 10x and 15x tape-stripping for 5 consecutive days resulted mainly in parakeratosis 72 h or later after the first tape-stripping moment. The 5x repeated tape-stripping stimulus did not result in parakeratosis. Scoring of parakeratosis based on RCM and histology corresponded well (Figure 4). Although loss of a honeycomb pattern, indicating epidermal atypia, and spongiosis could be observed with RCM, no correlation was found between this measure and epidermal proliferation, thickness or other parameters (Figures 4g and h).

Absence of epidermal inflammation
Influx of highly reflective inflammatory cells into the epidermis was not observed by RCM imaging. This corresponded very well with the observations in the CD3 and Elastase IHC stained tissue sections, since no inflammatory cells were found in the epidermis. IHC staining showed some influx of PMN and T-cells in the dermis (Table 1, Supplementary Figure). Unfortunately, limitations in penetration depth and epidermal thickening did not allow visualization of these cells with RCM.

Vascularization in relation to the basal layer and papillae
At 24 h after the acute tape-stripping challenge, the CD31 positive area in the dermis increased to some extent (Supplementary Figure). For the 10x and 15x repeated tape-stripping stimulus, this increase was observed after 72 h. Again, 5x repeated tape-stripping did not result in differences over time. In order to define a RCM measure for vascularization, the number of papillae were counted. Unfortunately, this was only possible in a low number of volunteers, which did not allow to determine differences in the number of papillae between the groups and over time. This ensured that no reliable correlation analysis between the number of papillae and CD31+ area in the dermis or number of proliferating cells could be performed.

Acute, 10x repeated and 15x repeated tape-stripping, resulted in a small increased ratio of basement membrane membrane length over SC length (BM/SC ratio) over time. Although a trend could be observed, significant differences between all tape-stripping stimuli were not found. In addition, 5x tape-stripping did not result in changes in the BM/SC ratio. The calculated ratio did not correlate to the number of proliferating cells (Pearson r=0.013) or area positive for CD31 staining (Pearson r=0.05).

Figure 4
RCM images in the horizontal plane and pictures of hematoxylin-eosin (HE) stained vertical sliced tissue sections of normal skin, parakeratosis, epidermal atypia and mild spongiosis. a) RCM image at the level of the stratum corneum at which no nucleated cells can be seen. b) At the level of the stratum granulosum, the first nucleated cells appear as dark rounds with a brighter halo representing the nucleus with the surrounding cytoplasm. c) Conventional HE stained tissue section of normal skin. Showing a thin stratum corneum, with no nucleated cells. The keratinocytes in the stratum spinosum are similar in appearance and are ordered in a regular honeycomb pattern. d) RCM image at the level of the stratum corneum, small highly reflective bright particles are visible representing parakeratosis. e) Due to the thickening of the stratum corneum the stratum granulosum is not visible at the same level as in normal skin. This image shows the presence of bright nucleated cells within the stratum corneum, indicating parakeratosis. f) HE stained tissue section of parakeratotic skin. The stratum corneum is thickened and nucleated cells are present within this layer (right corner). g) RCM image of normal skin at the level of the stratum spinosum, showing a regular honeycomb pattern. h) 72 h after acute skin damage, epidermal atypia is visible represented by the loss of the honeycomb pattern and some bright intracellular spaces are visible representing mild spongiosis. i) HE stained tissue section showing epidermal atypia. Keratinocytes differ in size and shape and are surrounded by some spongiosis.
Discussion

We studied the value of the tape-stripping model combined with in vivo RCM imaging as a non-invasive method to investigate skin damage and recovery over time after mechanical skin-material interaction. The induced skin changes were followed with multiple techniques to validate non-invasive RCM imaging as a technique in this field. RCM images were correlated with conventional (immuno)-histopathology in order to provide evidence for a non-invasive model for future use.

Thickness as measured in RCM images showed a strong correlation with the thickness as seen in histology for both SC and living epidermis. Thus, RCM can be used as a non-invasive tool to measure epidermal thickness in vivo. Thickness measurements of the acute skin damage model are in line with previous reported data. With a small delay in time, repeated tape-stripping for 5 consecutive days with a frequency of 10x or 15x induced skin changes comparable to those observed after acute tape-stripping. For this reason the acute tape-stripping model may perhaps be useful in studying both acute and repeated skin damage. In contrast to 10x and 15x repeated tape-stripping, mild tape-stripping (5x) for 5 consecutive days did not result in skin changes, indicating a threshold value for skin recovery and development of skin damage.

Interestingly, the in vivo epidermal thickness measurements with RCM correlated well to the number of Ki67-positive cells as seen in histology, thus representing a non-invasive in vivo measure for epidermal proliferation. The observed expression of the hyperproliferation markers Ki67 and cytokeratin 16 in the used models are in line with previous described data on tape-stripping. Although a small reduction of keratinocyte proliferation was observed 24 h after acute tape-stripping, we did not find significant differences between the time points. We therefore, were not able to confirm the hypothesis made by Hendriks et al that the mechanical stressor transiently paralyzes the basal keratinocytes after 24 h.

Besides epidermal proliferation and abnormal differentiation, parakeratosis (a measure for wound healing) can also be very well visualized with RCM. Keratinocyte atypia may be present in case of disturbed proliferation. Other processes like stress, environmental factors or skin diseases can also induce keratinocyte atypia and concurrent spongiosis, explaining the variety in the presence of epidermal atypia in the evaluated skin damage model. However, when present, RCM imaging allows visualization of epidermal atypia and spongiosis.

Measurement of transepidermal water loss (TEWL) and erythema are other non-invasive techniques used in the tape-stripping model to study skin barrier function indirectly. Erythema is primarily a subjective method, which is therefore a limiting measure in skin damage research. Unfortunately, with RCM it was not possible to determine a non-invasive objective method for vascularization, which might have been linked to the degree of erythema in future. Although some limitations of TEWL measurements have been reported, combining TEWL and erythema measurements with the currently evaluated non-invasive skin damage model will complement the model by supplying biophysical data in addition to the morphological RCM data. A combination with self-assessment to measure discomfort is important to determine the user friendliness of products and materials.

It should be mentioned that mechanical provocation of the skin not only involves the epidermis. Forces and velocity play also an important role in the caused damage, and are recommended to evaluate in future studies.

In conclusion, the combination of tape-stripping with RCM imaging can be used as a non-invasive model to obtain in vivo morphological data on skin-material interactions. In order to improve and develop skin friendlier materials and products, this in vivo model can be used in skin-material testing for exposure to repeated or acute impact. Epidermal thickness, keratinocyte proliferation, parakeratosis, epidermal atypia and spongiosis are parameters that can be studied over time.
CHAPTER 4

References


4. Bergboer JWT, Tjabringa GS, Kamsteeg M et al. Psoriasis risk genes of the late cornified envelope-3 group are distinctly expressed compared with genes of other lce groups. Am J Pathol 2011;178;1470-1477.


Supplementary figure

Representative pictures of K16, Basalec, CD3, and CD31 immunohistochemical staining over time after acute tape-stripping.
Summary and general discussion
The skin is a complex part of the human body and many diseases and processes are related to this organ. In chapter 1 it was described that skin cancer is a raising problem and therefore the need for non-invasive diagnostic tools is increasing. In addition, dynamic processes in the skin are difficult to study on static histological images, obtained by an invasive biopsy. Exploring the morphology of skin damage is also limited by the need of invasive biopsies. Therefore, the goal of this thesis was to study innovative applications for non-invasive in vivo reflectance confocal microscopy (RCM) in the field of skin cancer diagnosis, skin inflammation and skin damage.

In skin cancer diagnosis, RCM is mainly applied in melanocytic lesions. However, in the field of NMSC not all applications are carefully evaluated. Chapter 2 covers the survey in using RCM in different NMSC lesions.

In chapter 2.1 we defined RCM features for in vivo distinction between nodular, micronodular and superficial basal cell carcinoma (BCC). Tumor nests (aggregations of basaloid cells) with peripheral palisading, branch-like structures, fibrotic septa surrounding the tumor nests and increase in vascular diameter were the main characteristics for nodular and micronodular BCC. The size, shape and location of the tumor nests allows further distinction between those two subtypes of BCC. Solar elastosis and tumor nests located just below or in connection with the basal cell layer characterizes superficial BCC. The findings on superficial BCC and nodular BCC were recently confirmed by a study performed by Longo et al., but they did not make a distinction between nodular BCC and micronodular BCC, which is however important for determining the margin for surgical excision. Unfortunately in our study, features for infiltrative BCC could not be determined since our study only included an infiltrative BCC, which was a mixed type BCC, with the infiltrative component deeper in the dermis. Longo et al. nicely described RCM features for infiltrative BCC. However, RCM users should be aware that infiltrative BCCs are challenging to visualize due to the histological complex appearance and deeper location of the tumor nests in the dermis. This together with resolution limitations in depth can result in differences between confocal images of infiltrative basal cell carcinomas.

As presented in chapter 2.2, clinical differentiation between nodular BCCs and benign intradermal nevi can be difficult. RCM appears to be of value in establishing the correct diagnosis in a prospective way. The observed RCM features were in line with features for nodular BCC or intradermal nevi described in literature. Tumor nests of basaloid cells with peripheral palisiding, bright filaments and surrounding clefts are RCM features specific for nodular BCC. In contrast, a ringed or clod pattern at the dermal-epidermal junction (DEJ) and dermal dense-sparse nests were specific for intradermal nevi.

Squamous cell carcinoma (SCC) and actinic keratosis (AK) lesions can also be similar in their clinical appearance, therefore early diagnosis of SCC and distinction between SCC and AK is challenging. In chapter 2.3 we report RCM features to distinguish between these two kinds of skin lesions non-invasively. The presence of architectural disarray in the stratum granulosum in combination with architectural disarray in the stratum spinosum
and/or tumor nests in the dermis were main RCM features to differentiate between SCC and AK.4,6 RCM is a promising technique in differentiation between pre-malignant and malignant lesions and might prevent unnecessary biopsies of benign or pre-malignant lesions.

Besides the challenge in diagnosis of clinical similar appearing lesions, previous biopsied and treated lesions can also be difficult to clinically evaluate and diagnose. **Chapter 2.4** describes the clinical application of RCM as tool to select the location for a punch biopsy in a large and clinically indistinctive, previous treated and biopsied lesion. A great advantage of RCM is its ability to evaluate a total lesion, which might reduce sample errors and delay in accurate diagnosis and treatment. This demonstrates the value of RCM in the dermatological patient care.

Dynamic processes in the skin are important in understanding the pathogenesis of inflammatory skin diseases. In **Chapter 3** we report the findings on using non-invasive RCM in monitoring dynamic inflammatory processes in vivo.

**Chapter 3.1** presents our findings on the dynamics of neutrophil migration in psoriatic lesions. A cyclic pattern of neutrophil migration was observed. This consisted of neutrophil extravasation out of the dermal papilla, epidermal migration through the stratum spinosum towards the stratum corneum, accumulation in the stratum spinosum, accumulation in the stratum corneum and phases of microabscesses degeneration, confirming the findings based on static histopathological data published by Pinkus et al.7 In addition, we describe that the duration and time phasing of the neutrophil trafficking cycle was 5-7 days and that the morphology of these cells changes during the several phases. At the dermal-epidermal junction (DEJ), neutrophils start as highly reflective round to oval shaped cells. While trafficking towards the stratum corneum, the neutrophils were less round and more polyform, very bright and large. Accumulation of neutrophils in the upper epidermis appears as an aggregation of bright cells surrounded by a dark non-cellular area. Degeneration of the abscesses was recognized by decrease in brightness resulting in a homogenous grey area, which later on became dark with only a few bright-scattered neutrophil rests visible.

As the accumulation of neutrophils in diseased skin is a random process, the use of the leukotriene B4 (LTB4) skin inflammation model in combination with RCM was described in **Chapter 3.2**. Epicutaneous application of LTB4 results in epidermal accumulation of polymorphonuclear neutrophils (PMN), with known time intervals. Based on RCM images, we report that the appearance of the PMN throughout the stratum spinosum changed from solitary small round to oval bright particles at the level of the DEJ to highly reflective ill-defined cells with a granular content at the level of the stratum granulosum, representing the changes of PMN during migration, also described in psoriatic lesions.2 RCM allows imaging of the accumulation and degeneration of these PMN over time. Accumulated in the stratum corneum, PMN seem to vibrate to some extent. Most likely, this vibration on the real-time images is caused by the complexity of these poly-nucleated cells. The light reflected by these complex and large cells will be more scattered compared to cells with a small round nucleus. It might also be possible, that these cells really vibrate to some extent, as they are trafficking through the epidermis. Although epidermal thickening as result of LTB4 application is a known phenomenon,1,10 to the best of our knowledge, we are the first who demonstrated the accurate correlation between epidermal thickness measured on histological sections and RCM images. RCM in combination with the LTB4 induced skin inflammation seems to be promising in studying and monitoring dynamic inflammatory processes in the skin.

Skin comfort is becoming more important in developing materials that interact with the skin. In **Chapter 4** the changes caused by skin-material interactions were evaluated and a non-invasive model for this phenomenon was developed.

**Chapter 4.1** provides us with (immuno)-histological data after a sliding on artificial turf and natural grass. Clinically, dry natural grass causes more erythema, while a sliding on artificial turf results in abrasion. At morphological level, the stratum corneum is removed after a sliding on dry natural grass or artificial turf. Further, a sliding on these two pitches changes epidermal differentiation and the skin barrier function. Wet natural grass on the other hand, does not result in removal of the stratum corneum or changes in epidermal differentiation and skin barrier function. These morphological data demonstrate that many changes in the skin cannot be scored at the outside. Therefore, in studying skin-material interactions, the skin in total should be used as read-out system.

Since invasive skin biopsies are difficult to obtain in studying skin-material interactions, in **Chapter 4.2** we report about the use of tape stripping and RCM as in vivo model to study skin damage. A strong correlation between histology results and RCM images for both stratum corneum and living epidermal thickness measurement was found. It was concluded that epidermal thickness assessed by RCM can be used as a measure for keratinocyte proliferation in vivo. When present, RCM can visualize parakeratosis, epidermal atypia and spongiosis in this model. Inflammatory cells did not infiltrate the epidermis and were therefore not observed by RCM. Repeated 10 or 15x tape stripping on the lower back skin for 5 consecutive days shows, with a small delay in time, a similar response as the acute tape stripping stimulus. In contrast, 5x tape stripping for 5 consecutive days seem to be a threshold value for development of skin damage on the lower back skin. These findings show that the combination of tape stripping with RCM imaging might be used as a non-invasive skin damage model (repeated and acute impact exposure) to obtain in vivo morphological data on skin-material interactions. This might allow to improve and develop skin friendlier materials and products.
General discussion

Non-invasive imaging of the skin is becoming of great value in dermatological patient care as well as in the development of skin friendly products and materials, resulting in skin comfort, efficient diagnosis and proper treatment. RCM, a non-invasive imaging technique, has proven to be promising in diagnosing skin cancer and inflammatory skin diseases.\textsuperscript{11, 12} RCM features for different kind of skin lesions are described and we have shown the ability of RCM to distinguish between the different types of NMSC. However, for implementation of this technique in the Dutch patient care, prospective large cohort randomized control trails are required. Until now, most studies are descriptive and there are few studies performed to determine the sensitivity and specificity of diagnosing melanoma and basal cell carcinoma by RCM. However, most results are obtained in a retrospective way and do not evaluate all (sub)types of skin cancers.\textsuperscript{1, 18, 19} A prospective study, which will include all skin cancer types, will allow to determine the exact value of this promising non-invasive imaging technique for the dermatological skin cancer care. As such a study might result in developing protocols and guidelines, this will contribute towards implementation of RCM in the Dutch health care system. Currently, two other Dutch hospitals have purchased the RCM device, demonstrating the increasing interest in non-invasive diagnosis of skin cancer lesions. Further, this points out the need for protocols and guidelines in order to get a standardized way of RCM measurements and interpretation of the images.

Besides in diagnosing skin lesions, RCM has been proven to be a useful technique for the follow-up of lesions or for treatment success.\textsuperscript{11, 20 -22} Further, we have demonstrated the promising application of RCM in unraveling dynamic processes in vivo, which has been confirmed by Moscarella et al.\textsuperscript{23} This indicates that for future, in trials on new drugs, it may not always be necessary to obtain biopsies to test the efficacy and safety of new (non-invasive) therapies at a morphological level.

As described in this thesis, RCM is a useful technique in diagnosing skin lesions, follow-up of therapy and unraveling pathophysiology of skin diseases. However, also other non-invasive diagnostic imaging techniques have been developed and may be promising in these fields. Conventional optical coherence tomography (OCT) provides a real-time, cross-sectional, vertical representation of tissue, however the lateral resolution is only about 10 to 15 µm. Therefore, high-definition OCT scanners have been developed for medical applications providing significant higher resolution (1-3 µm) than conventional OCT techniques. Although improved, the resolution of high-definition OCT is still lower than the lateral resolution of RCM (0.5-1 µm), which is important for distinguishing sub-cellular structures. In contrast, OCT has a higher penetration depth compared to RCM. The clinical application of high-definition OCT over conventional OCT is promising, but large cohort studies have to be performed to demonstrate the efficiency of this technique in the field of dermatology.\textsuperscript{24 -27} Multiphoton fluorescence microscopy (MFM) is a high-resolution laser scanning imaging technique enabling deep optical imaging of tissues. Cellular and sub-cellular resolution can be provided by this technique. With RCM and OCT, the light has to be focused on a small spot and spatial filters such as pinholes avoid detection of photons from out-of-focus regions. MFM on the other hand uses a pulsed laser beam to realize nonlinear non-confocal high-resolution luminescence imaging based on an excitation volume (1µm³), detecting auto-fluorescent components. Both cells and extracellular matrix intrinsically contain a variety of fluorescent molecules (NADH, tryptophan, keratin, melanin, elastin, cholecalciferol), these biological tissues can be imaged without any exogenously added probe.\textsuperscript{28, 29} Compared to RCM and high-definition OCT, MFM has a higher spatial resolution. MFM has been applied on ex vivo tissue samples\textsuperscript{30} and in vivo human skin,\textsuperscript{31 -33} but there is a large field of undiscovered applications of this interesting new non-invasive skin imaging technique. It would be interesting to compare RCM, high-definition OCT and MFM in order to determine the value of each technique for dermatological patient care, research and industry.

As skin comfort is becoming a major topic in the development of products that interact with the skin, there is an increasing need for morphological knowledge about skin-material interactions. Until now, morphological evaluation of the skin required an invasive skin biopsy. In research and material testing, obtaining these biopsies is ethically limited and moreover, these biopsies induce inflammation by itself, which interfere with the study results. This restricts to explore morphological changes after skin-material interaction. Non-invasive imaging techniques such as RCM, high-definition OCT and MFM are therefore promising techniques in this field. Besides efficient, patient friendly diagnostic tools for the dermatological patient care, application of these techniques will contribute to development of user friendlier materials and products.

With the increasing skin cancer incidence and attention for skin comfort, we are convinced that non-invasive imaging of the skin is becoming more important as a patient and user friendly approach. Non-invasive imaging techniques will help to manage the increasing skin cancer problem, will help to unravel pathophysiologically processes, and will improve morphological knowledge on skin-material interactions in order to develop skin friendly products.
References


Nederlandse samenvatting
Samenvatting

De huid is een complex onderdeel van het menselijk lichaam en vele ziekten en processen zijn gerelateerd aan dit orgaan. In hoofdstuk 1 is beschreven dat huidkanker een toenemend gezondheidsprobleem is. De gouden standaard qua diagnostiek is pathologisch onderzoek van een huidbiopt. Deze techniek heeft naast de invasiviteit ook andere nadelen. Een belangrijk nadeel is dat er altijd sprake van sampling is, wat kan leiden tot een sampling error. Er wordt in dit geval een verkeerde diagnose gesteld met alle consequenties van dien. Daarnaast is het moeilijk om met de conventionele diagnostiek dynamische processen in de huid in de loop van de tijd te bestuderen aan de hand van statische histologische beelden. Naast het feit dat de procedure patiëntvriendelijk is, induceren de huidbiopten door de invasiviteit bovendien een wondje en een inflammatoire respons, wat de resultaten van een onderzoek kan beïnvloeden. De noodzakelijkheid van biopten is tevens een limiterende factor in het ontrafelen van de morfologie van huidschade. Er is dus een toenemende vraag naar niet-invasieve diagnostische technieken binnen de dermatologie. Het doel van het onderzoek beschreven in dit proefschrift was mogelijke toepassingen van niet-invasieve reflectie confocale microscopie (RCM) op het gebied van huidkanker en ontstekingsprocessen te bestuderen. Tevens was het doel om niet-invasieve modellen voor huidontsteking en huidschade te ontwikkelen en valideren.

Er is al veel bekend over RCM diagnostiek bij melanomen, maar met name de toepassingen op het gebied van niet-melanoma huidkanker zijn nog niet volledig in kaart gebracht. Hoofdstuk 2 bevat de bevindingen over het gebruik van RCM op het gebied van niet-melanoma huidkanker.

In hoofdstuk 2.1 hebben we RCM kenmerken beschreven die het mogelijk maken om in vivo onderscheid te maken tussen nodulair, micronodulair en superficieel basaalcellencarcinoom (BCC). De meest karakteristieke RCM kenmerken voor nodulair en micronodulair BCC zijn tumornesten (aggregatie van basaloide cellen in de dermis) met perifere palisadering, hoogreflectieve vertakkingen in de tumornesten, omliggende fibrotische septa en toegenomen vascularisatie. De grootte, de vorm en de locatie van de tumornesten maken verder onderscheid tussen nodulair en micronodulair BCC mogelijk. Solaire elastose in de dermis en tumornesten die direct onder of in verbinding met de basale cellaag zijn gelokaliseerd, zijn RCM kenmerken voor superficiële BCC's. Het maken van onderscheid tussen subtypen van BCC door middel van RCM draagt bij aan het direct kunnen bepalen van de therapie en excisie marges, op een niet-invasieve manier. Zoals beschreven in hoofdstuk 2.2, kan het lastig zijn om klinisch onderscheid te maken tussen nodulaire BCCs en benigne intradermale naevi. RCM blijkt van toegevoegde waarde in het correct prospectief diagnosticeren van deze twee typen laesies. Tumornesten met perifere palisadering, hoogreflectieve structuren en omringende donkere spleten zijn specifiek voor een nodulair BCC. Een ringpatroon op de dermale-epidermale overgang en compacte verspreidde nesten daarentegen zijn specifiek voor
intradermale naïeves. Deze kenmerken lijken specifiek genoeg om op een prospectieve manier onderscheid te maken tussen nodulaire BCCs en intradermale naïeves die er klinisch hetzelfde uitzien.

Plaviescelcarcinoom (PCC) en actinische keratose (AK) kunnen net als een nodular BCC en intradermale naïeves overeenkomsten in het klinische beeld vertonen. In hoofdstuk 2.3 hebben we daarom RCM kenmerken beschreven die onderscheid kunnen maken tussen PCC en AK. De aanwezigheid van een onregelmatige architectuur in het stratum granulosum gecombineerd met een onregelmatige architectuur in het stratum spinosum en/of dermaal gelokaliseerde tumormesten zijn de belangrijkste kenmerken die onderscheid kunnen maken tussen PCC en AK. Correcte diagnose van laesies die er klinisch hetzelfde uitzien, zal onnodige biopten van benigne of premaligne laesies voorkomen.

Hoofdstuk 2.4 beschrijft het gebruik van RCM als tool in het selecteren van een geschikte locatie voor het nemen van een huidbiopsie in een grote, eerder behandelde en gebiospteerd, klinisch lastig te beoordelen laesie. Dit laat de toegevoegde waarde van RCM zien in de dermatologische patiëntenzorg. RCM heeft als voordeel een gehele laesie te kunnen afscanen wat de kans op een sampling error verkleint en een vertraging van te kunnen afscannen wat de kans op een sampling error verkleint en een vertraging van bedside biopsy kan voorkomen.

Dynamische processen in de huid zijn belangrijk om de pathogenese van inflammatoire huidziekten te begrijpen. In hoofdstuk 3 beschrijven we het gebruik van niet-invasieve RCM voor het monitoren van dynamische inflammatoire processen in vivo.

Hoofdstuk 3.1 laat onze bevindingen zien over de dynamiek van neutrofielen migratie in psoriasis laesies. Deze migratie treedt op in een cyclisch patroon en bestaat uit het uittreden van de neutrofielen uit de dermale papillen, het migreren door het stratum spinosum richting het stratum corneum, het accumuleren in het stratum spinosum en stratum corneum en de uiteindelijke degeneratie van de ontstane microabcessen. De duur van deze migratiecyclus is 5 tot 7 dagen en de morfologie van de neutrofielen verandert gedurende de verschillende fasen van de cyclus. Op de dermale-epidermale overgang zijn de neutrofielen hoog reflectieve ronde/ovale cellen. Gedurende de migratie door het stratum spinosum tot aan het stratum corneum veranderen. Op de dermale-epidermale overgang zijn deze cellen hoogreflectief maar minder scherp omgrenst en meer granulair. Hoog in de epidermis lijken deze cellen iets te bewegen, passend bij de complexiteit van de cellen of het migratieproces. Naast de accumulatie van PMNs in dit model, is tevens de degeneratie van deze cellen te visualiseren. De dikte van de epidermis neemt na applicatie van LTB4 toe gedurende de tijd, deze dikte is goed te meten met RCM. Deze bevindingen laten zien dat RCM in combinatie met het LTB4 model of met laesionale psoriasis huid veelbelovend is om dynamische inflammatoire processen in de huid in vivo te bestuderen en monitoren.

Huidcomfort wordt steeds belangrijker in de ontwikkeling van materialen en producten die een interactie aangaan met de huid. In hoofdstuk 4 zijn de veranderingen die ontstaan door huid-materiaal interactiviteiten geëvalueerd met als doel een niet-invasief model te ontwikkelen voor dit soort interactiviteiten.

Hoofdstuk 4.1 geeft de (immuno)-histologische veranderingen na een sliding op kunstgras en natuurgras weer. Klinisch is er met name erytheem zichtbaar na een sliding op droog natuurgras en resulteert een sliding op kunstgras in een schaafwond. Op morphologisch niveau is het stratum corneum verwijderd na zowel een sliding op kunstgras als droog natuurgras. Daarnaast zorgt een sliding op deze twee soorten velden voor een verandering in epidermale differentiatie en de huid barrièrefunctie. Natuurgras resulteert niet in de verwijdering van het stratum corneum en veranderingen in epidermale differentiatie en de huid barrièrefunctie. Deze morfologische data laten zien dat er meer gebeurt in de huid dan dat aan de buitenkant kan worden geëvalueerd. Dit demonstreert dat de huid in zijn geheel gebruikt dient te worden als uitleessysteem voor de huidschade ontstaan door huid-materiaal interactiviteiten.

Invasieve huidbiopten kunnen meerder niet worden afgemengen in onderzoeken naar huid-materiaal interactiviteiten, daarom beschrijven we in hoofdstuk 4.2 het gebruik van tapestrippen in combinatie met RCM als in vivo model om huidschade te bestuderen. De metingen van de dikte van de epidermis en de huidbarrièrefunctie werden met een sliding op kunstgras en natuurgras gecompliceerd met het LTB4 model. Herhaalde tape strippen, 10 of 15x, gedurende 5 aaneengesloten dagen zorgde voor een indicatie voor de mate van epidermale proliferatie. Met RCM konden granulair en histologisch onderzoek. Tevens werd aangetoond dat deze metingen van de epidermis een indicatie zijn voor de mate van epidermale proliferatie. Met RCM konden in dit model ook parakeratose en keratinocytagyatie worden waargenomen. Inflammatoire cellen infiltreren de epidermis niet en konden hierdoor ook niet worden gevisualiseerd met RCM. Herhaalde tape strippen, 10 of 15x, gedurende 5 aaneengesloten dagen zorgde voor een indicatie voor de mate van epidermale proliferatie. In de dermale-epidermale overgang zijn losse, kleine, ronde hoogreflectieve cellen zichtbaar. In de RCM beelden is te zien dat de vorm en reflectie van de PMNs gedurende de migratie door het stratum spinosum tot aan het stratum corneum veranderen. Op de dermale-epidermale overgang zijn deze cellen hoogreflectief maar minder scherp omgrenst en meer granulair. Hoog in de epidermis lijken deze cellen iets te bewegen, passend bij de complexiteit van de cellen of het migratieproces. Naast de accumulatie van PMNs in dit model, is tevens de degeneratie van deze cellen te visualiseren. De dikte van de epidermis neemt na applicatie van LTB4 toe gedurende de tijd, deze dikte is goed te meten met RCM. Deze bevindingen laten zien dat RCM in combinatie met het LTB4 model of met laesionale psoriasis huid veelbelovend is om dynamische inflammatoire processen in de huid in vivo te bestuderen en monitoren.
en acute blootstelling) om morfologische gegevens over huid-materiaal interacties te verkrijgen. Deze kennis zal bijdragen aan het verbeteren en ontwikkelen van huidvriendelijke materialen en producten.

Niet-invasieve beeldvorming van de huid wordt steeds belangrijker door de toenemende incidentie van huidkanker en de aandacht voor huidcomfort. In vivo beeldvormende technieken kunnen helpen om dit toenemende gezondheidsprobleem te managen en kunnen bijdragen aan het vergroten van de morfologische kennis over huid-materiaal interacties met als doel het ontwikkelen van huidvriendelijke producten.
List of publications
Curriculum Vitae
Dankwoord
List of publications

Publications related to this thesis


* These authors contributed equally
Curriculum Vitae


Publications not related to this thesis


Dankwoord

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Malou
### List of abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AK</td>
<td>Actinic keratosis</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>BD</td>
<td>Bowen disease</td>
</tr>
<tr>
<td>D</td>
<td>Dermis</td>
</tr>
<tr>
<td>DEJ</td>
<td>Dermal-epidermal junction</td>
</tr>
<tr>
<td>FDAP</td>
<td>Fluorescence diagnosis with aminolevulinic acid-induced porphyrins</td>
</tr>
<tr>
<td>hBD-2</td>
<td>Human beta defensin-2</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin-eosin</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>iBCC</td>
<td>Infiltrative basal cell carcinoma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemical</td>
</tr>
<tr>
<td>κ</td>
<td>Kappa</td>
</tr>
<tr>
<td>K10</td>
<td>Cytokeratin 10</td>
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<tr>
<td>K16</td>
<td>Cytokeratin 16</td>
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<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
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<tr>
<td>MFM</td>
<td>Multiphoton fluorescence microscopy</td>
</tr>
<tr>
<td>mnBCC</td>
<td>Micronodular basal cell carcinoma</td>
</tr>
<tr>
<td>nBCC</td>
<td>Nodular basal cell carcinoma</td>
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<tr>
<td>NMSC</td>
<td>Non-melanoma skin cancer</td>
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<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
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<tr>
<td>PpIX</td>
<td>Photosensitizer protoporphyrin-IX</td>
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<tr>
<td>RCM</td>
<td>Reflectance confocal microscopy</td>
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<tr>
<td>sBCC</td>
<td>Superficial basal cell carcinoma</td>
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<td>SC</td>
<td>Stratum corneum</td>
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<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<td>SG</td>
<td>Stratum granulosum</td>
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<td>SS</td>
<td>Stratum spinosum</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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