Selected aspects on improving
the management of skin cancer

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Playa de la Concha, San Sebastián, Spain. Photo: John Paoli.

"… doing what little one can to increase the general stock of knowledge

is as respectable an object of life, as one can in any likelihood pursue"

\- Charles Darwin

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ABSTRACT

The constant rise of skin cancer incidence rates in Sweden is a problem which requires attention. Improved techniques for prevention, early detection and effective treatment are required to face this challenging task. The studies presented in this thesis deal with selected aspects concerning diagnostic, therapeutic and preventive methods with the aim of improving the management of skin cancer.

The diagnosis of skin cancer today is mainly based on visual examination, dermoscopy and histopathology, but several new imaging techniques are under development. In this thesis, multiphoton laser scanning microscopy (MPLSM) was used to study non-melanoma skin cancers (NMSCs) in comparison to healthy skin *ex vivo*. Typical histopathological criteria were observed on a subcellular level in superficial basal cell carcinomas and squamous cell carcinoma *in situ* lesions. However, the limited imaging depth of approximately 100 μm made imaging of thicker nodular basal cell carcinomas more difficult.

One effective therapeutic option for superficial NMSCs is photodynamic therapy (PDT). It is considered a first-line therapy for extensive areas of actinic keratoses (AKs) and new indications are being evaluated. Penile intraepithelial neoplasia (PIN) lacks effective treatments with low recurrence rates. The effectiveness of PDT in the treatment of PIN was studied. Seven out of ten patients responded to treatment and four showed no recurrences after a mean follow-up of 35 months. Another issue is pain during PDT, a drawback with which physicians have struggled for years. A split-face study on patients with extensive AKs in the facial area showed that nerve blocks provided excellent pain relief during PDT.

Primary and secondary prevention of skin cancer involves campaigns that encourage sensible sun-exposure behaviors and promote skin self-examinations for early detection. As part of this thesis, the results of the 'Euromelanoma Day' screening campaign in Sweden 2008 were compiled. The detection rates of NMSC and malignant melanoma (MM) among the 2659 screened patients were up to 2-3 times higher than similar campaigns in other European countries. The prognosis of the 24 diagnosed MMs was predominantly favorable.

In conclusion, today's diagnostic, therapeutic and preventive measures have room for further development. New non-invasive imaging techniques like MPLSM may lead to bedside histopathological confirmation of a skin cancer diagnosis. PDT may be a therapeutic alternative for PIN and pain during PDT in the facial area can be effectively relieved with nerve blocks. Screening campaigns can obtain high detection rates of skin cancer when directed towards a population with high incidence rates.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the following text by their corresponding Roman numerals:

I.   John Paoli, Maria Smedh, Ann-Marie Wennberg and Marica B. Ericson. Multiphoton Laser Scanning Microscopy on Non-Melanoma Skin Cancer: Morphologic Features for Future Non-Invasive Diagnostics. *J Invest Dermatol* 2008; 128: 1248-55

II. John Paoli, Annika Ternesten Bratel, Gun-Britt Löwhagen, Bo Stenquist, Ola Forslund and Ann-Marie Wennberg. Penile Intraepithelial Neoplasia: Results of Photodynamic Therapy. *Acta Derm Venereol* 2006; 86: 418-421

III. John Paoli, Christina Halldin, Marica B. Ericson and Ann-Marie Wennberg. Nerve blocks provide effective pain relief during topical photodynamic therapy for extensive facial actinic keratoses. *Clin Exp Dermatol* 2008; 33: 559-564

IV. John Paoli, Markus Danielsson and Ann-Marie Wennberg. Results of the "Euromelanoma Day" Screening Campaign in Sweden 2008. Accepted for publication in the *J Eur Acad Dermatol Venereol* (April 6th, 2009).

CONTENTS

ABBREVIATIONS

1PE	one-photon excitation
2PE	two-photon excitation
5-FU	5-fluorouracil
AK	actinic keratosis
ALA	aminolevulinic acid
BCC	basal cell carcinoma
C&E	curettage and electrodesiccation
CO_2	carbon dioxide
DNS	dysplastic nevus syndrome
HPV	human papilloma virus
MAL	methyl aminolevulinate
MM	malignant melanoma
MMS	Mohs micrographic surgery
NADH	reduced nicotinamide adenine dinucleotide
MPLSM	multiphoton laser scanning microscopy
NIR	near infrared
NMSC	non-melanoma skin cancer
OCT	optical coherence tomography
OTR	organ transplant recipient
PDT	photodynamic therapy
PIN	penile intraepithelial neoplasia
PpIX	protoporphyrin IX
SCC	squamous cell carcinoma
SK	seborrhoeic keratosis
UV	ultraviolet
VAS	visual analog scale

1. INTRODUCTION

The incidence of skin cancer has constantly been rising the past decades in the Caucasian population around the world.[1-3] Sweden, with a population of approximately 9 million inhabitants, has one of the highest population-based incidence rates in Europe of the three main types of skin cancer: malignant melanoma (MM), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC).[4, 5] Today, more than 50 % of all patient visits to specialists in Dermatology and Venereology in Sweden are due to benign or malignant skin tumors. All skin cancers can potentially be cured if early detection and effective treatment can be provided. It is therefore crucial to find new strategies to improve the management of patients with skin cancer through:

- Development of quick, safe, cost-effective and reliable diagnostic and therapeutic techniques.
- Education of the general population and health care professionals in primary prevention and self-examination of the skin.
- Secondary prevention through further education and regular full body skin examinations of high-risk individuals.

The objective of the studies included in this thesis was to contribute to the goal of improving the diagnosis, therapy and prevention of skin cancer. A new imaging technique for the diagnosis of skin cancer is introduced in Paper I. Paper II presents a novel indication for a well-known topical treatment of superficial skin tumors. Paper III shows how pain relief during such therapy can be achieved using a simple anesthetic technique. Finally, the first results of a national skin cancer screening campaign in Sweden are presented in Paper IV.

1.1 The structure and function of human skin

Human skin, with a surface area of 1.5-2.0 m^2, is one of the largest organs of the human body.[6] It consists of three main layers: the epidermis, the dermis and the subcutis (Fig. 1). The most superficial of the three is the epidermis, which can be as thin as 0.05 mm on the eyelids and over 1 mm thick on the palms and soles.[7] The epidermis consists of several layers of cells named keratinocytes. These cells are constantly renewed through mitoses of the keratinocytes along the basal cell layer or *stratum basale*. They undergo a maturation process (keratinization) on their way through the overlying layers of the *stratum spinosum*, *granulosum* and *corneum* before they are finally shed off the skin surface as flat, scale-like cells. Among the keratinocytes of the *stratum basale*, there are dendritic cells known as melanocytes. These cells contain organelles known as melanosomes, which are responsible for the synthesis of a light-absorbing pigment called melanin, the skin's natural pigment. The production of melanin can be stimulated by hormones and ultraviolet (UV) radiation. Melanin is transported by the melanosomes to surrounding keratinocytes via the melanocyte's projections or dendrites to provide the skin with natural protection from the sun's UV light. Langerhans cells, which are part of the immune system, are also dendritic cells found in the epidermis. Besides these structures, the epidermis also clothes adnexal structures such as hair follicles with their adjacent sebaceous glands as well as eccrine and apocrine sweat glands, the bodies of which penetrate deeper into the skin. The epidermis' main role lies within the *stratum corneum*, a semipermeable barrier protecting the body from water loss.

The epidermis is separated from the underlying dermis by the basal membrane at the dermo-epidermal junction. In order to provide durability and elasticity to the skin, the dermis contains a mesh of fibers (*e.g.*, collagen, elastin, reticulin) produced by fibroblasts, which are submerged in a liquid ground substance composed mainly of water, glucosaminoglycans and hyaluronic acid. In the dermis there are also blood vessels, which provide the dermis and epidermis with nutrients and oxygen. The dermis is divided into the superficial papillary

dermis and the deeper reticular dermis by a superficial plexus of blood vessels. Capillaries extend from these vessels to the dermal papillae, which are regularly distributed extensions of the dermis into the epidermis, in order to nurture the epidermis. Lymphatic vessels, also present in the dermis, carry away excess waste products. The dermis' thickness varies between 1 and 10 mm, being thickest on the back.[7]

The deepest layer of the skin, the subcutis, mainly consists of fat-producing lipocytes, which is a reserve energy source. The subcutis also acts as thermal insulation and protects from trauma.

The skin is innervated by specialized sensory nerve endings allowing us to sense touch, pressure, vibration, temperature and pain. Motor innervation controls the activity of the hair-raising muscle (*m. arrectores pilorum*) and the sweat glands.
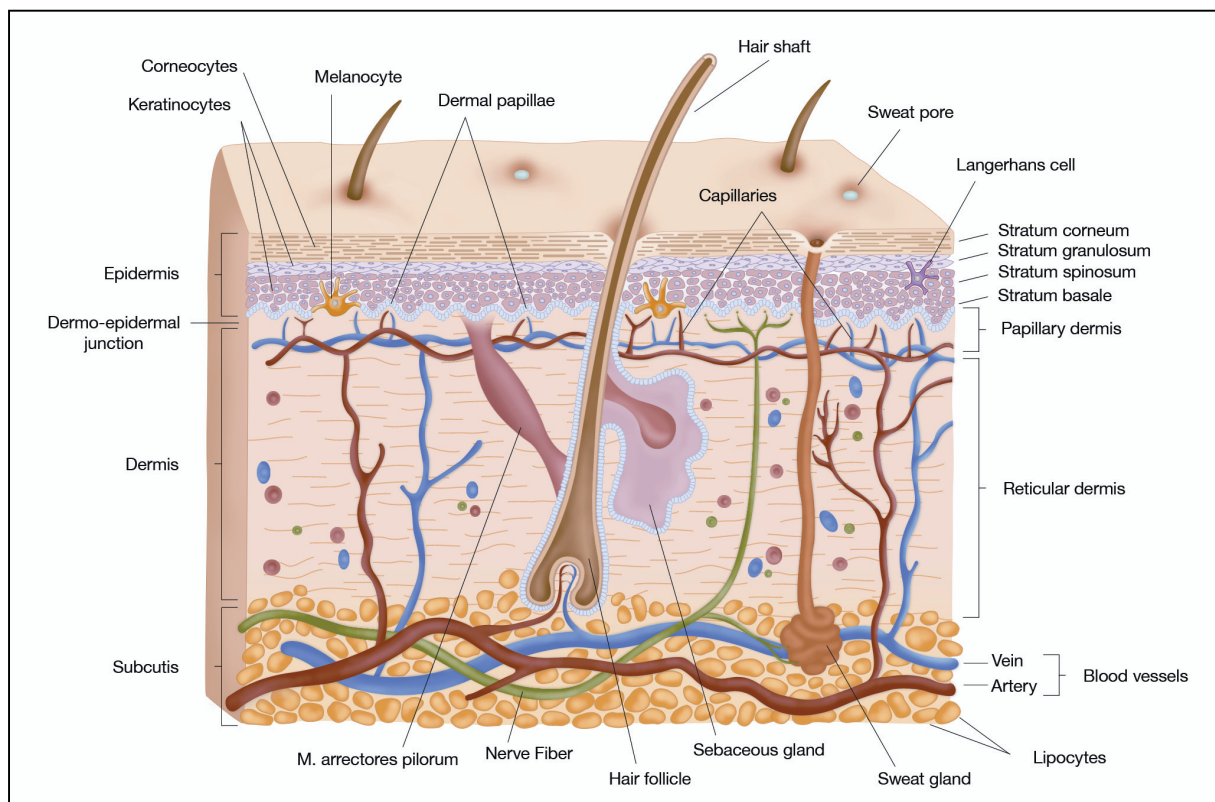
Figure 1. Skin anatomy. (Artwork: Ana Paoli)

1.2 Skin cancer

The most common types of benign and malignant lesions of the skin have their origin in melanocytes or keratinocytes. Typical melanocytic and non-melanocytic lesions (derived from melanocytes and keratinocytes, respectively) are shown in Figure 2.
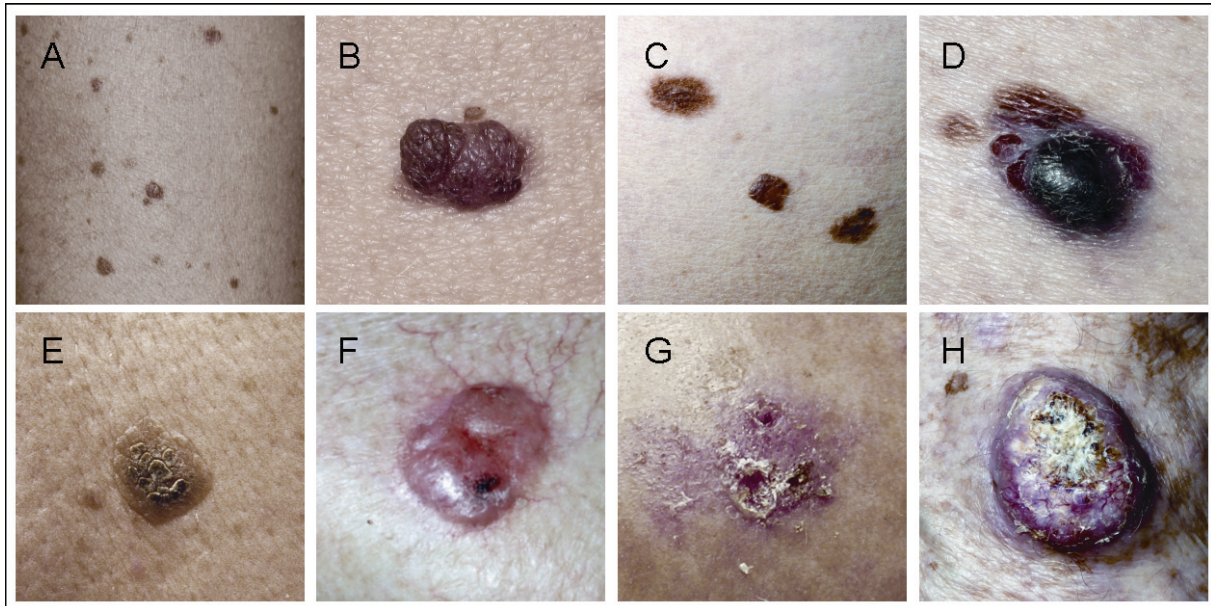
Figure 2. Common benign and malignant (A-D) melanocytic and (E-H) non-melanocytic lesions are shown: (A) multiple benign nevi, (B) a dermal nevus, (C) clinically atypical nevi, (D) a malignant melanoma, (E) a seborrhoeic keratosis, (F) a basal cell carcinoma, (G) an actinic keratosis with areas suspicious of SCC *in situ*, and (H) a squamous cell carcinoma. (Photo: John Paoli and Morgan Carlsson)

This thesis will concentrate on the three main kinds of skin cancer (MM, SCC and BCC). It should be noted that there are many other rare skin neoplasms (*e.g.*, dermatofibrosarcoma protuberans, atypical fibroxantoma or Merkel cell carcinoma), but these will not be covered here.

Skin cancer is one of the most common types of cancer in Caucasians.[4, 8, 9] In Australia, for example, an estimated 374 000 people were diagnosed with BCC or SCC in comparison with 92 876 patients with any other type of cancer in 2002.[8] The incidence and age-standardized incidence rates of MMs, SCCs and BCCs in Sweden are presented in Table 2.

Approximately the same amount of skin cancers are detected every year in Sweden compared to any other type of cancer.[4] The high incidence rates of invasive MM and SCC could make skin cancer, excluding BCCs, be considered the second most common type of cancer in both males and females after prostate and breast cancer respectively.[4] These statistics do not take into account the incidence of precursors to invasive MM (MM *in situ*) or SCC *in situ* (Table 1). The exact number of actinic keratoses (AKs, singular: actinic keratosis), the earliest precursor to SCC, is unknown but estimated to affect over 100 000 individuals in Sweden every year.

Table 1. New cases and age-standardized incidence rates (per 100 000 persons) of skin cancer in Sweden (population approximately 9 148 000 inhabitants) in 2007.[4]

Skin cancer type	New cases	Age-standardized incidence rate (males/females)	Annual increase last 10 years (males/females)
MM	2 333	26 / 24	3.6 % / 3.8 %
MM *in situ*	1 146	N.A.	N.A.
SCC	4 143	60 / 32	3.8 % / 5.3 %
SCC *in situ*	5 717	N.A.	N.A.
BCC*	39 133	415 / 336	N.A.

N.A., data not available; *, data from 2006, age-standardized incidence rates per 100 000 persons based on Sweden's population in 2000.[5]

1.2.1 Melanocytic lesions

Benign melanocytic lesions are commonly referred to as nevi (singular: nevus) and include: congenital nevi, common acquired melanocytic nevi (*e.g.*, lentigo simplex, junctional nevi, compound nevi, dermal nevi), dysplastic nevi and other more uncommon types (*e.g.* Spitz nevi, Reed nevi and blue nevi). Dysplastic nevi (syn. atypical nevi, Clark's nevi, B-K moles) were initially defined clinically as lesions with ill-defined pigmentation (different shades of brown, pink and/or red) and borders with a diameter larger than 5 mm.[10] Histopathologically, dysplastic nevi present intraepidermal lentiginous hyperplasia and random cytological atypia of the melanocytes in combination with a stromal response.[11] The clinical features correlate

poorly to the histopathological definition as cytological atypia is lacking in most "clinically atypical" nevi.[10, 12]

MMs are malignant melanocytic lesions which arise due to unregulated growth of melanocytes originating from a mutated melanocyte within a nevus or normal skin (*de novo*). In the most common types of MM, the malignant melanocytes initially grow radially within the epidermis (MM *in situ*) for an uncertain period of time before invading the dermis becoming an invasive MM with metastatic potential.[13] MMs are the most serious of the main types of skin cancer because they have a high potential for lymphatic and hematogenous spreading and, subsequently, a higher risk of causing mortality. Depending on the clinical and histopathological characteristics of the lesion, MMs can be classified into different histogenetic subtypes. The four most common are: superficially spreading melanoma, lentigo maligna melanoma, nodular melanoma and acral lentiginous melanoma. The precursor lesion (*in situ*) of lentigo maligna melanoma is called lentigo maligna. There are also rare types of melanoma such as: desmoplastic melanomas, malignant blue nevi, animal-type melanomas and nevoid melanomas.[14] Hypomelanotic or amelanotic MM is a subtype with little or no pigment, which can make the diagnosis more difficult. It has been estimated that approximately 2-8% of all MMs are partially devoid of pigment (hypomelanotic), whereas truly amelanotic MMs, completely lacking pigment, are rare.[15]

The prognosis of a MM is determined by the invasion depth, the presence or absence of ulceration, lymph node involvement (see sentinel lymph node biopsy in section 1.3.3) or distant metastases. Other prognostic factors which are currently being investigated are tumor vascularity, vascular invasion, mitotic rate, tumor regression, and tumor-infiltrating lymphocytes.[16] Invasion depth can be measured according to the Breslow thickness and/or the Clark level (Fig. 3).[17-19] Thus, thin MMs which have an invasion depth of less than 1 mm or a low Clark level (I-III) have a much more favorable prognosis in comparison with thick lesions which are more than 4 mm thick or have a Clark level IV-V, for example.

Breslow thickness   Clark level
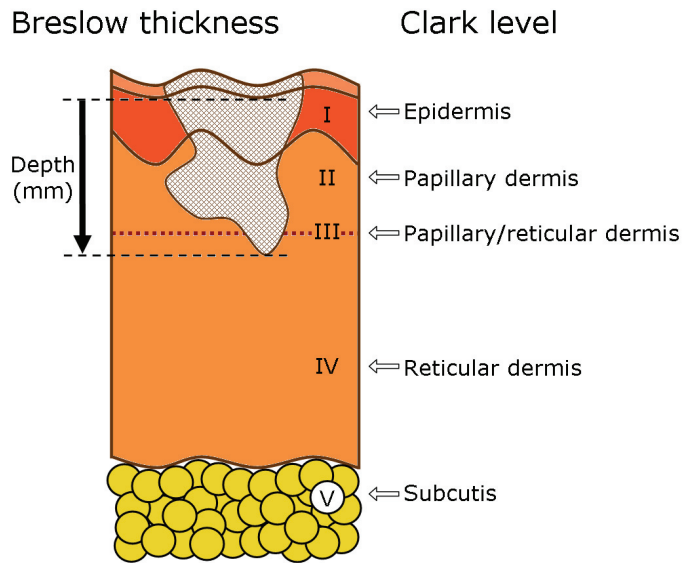
Figure 3. The Breslow thickness is measured in mm from the *stratum granulosum* down to the deepest lying invasive melanoma cell. Clark level I represents intraepidermal growth (*in situ*), level II describes invasion of the papillary dermis, level III implies filling of the entire papillary dermis, level IV means invasion of the reticular dermis and level V signifies invasion of the subcutis. (Artwork: J. Paoli)

1.2.2 Non-melanocytic lesions

There are a vast number of different benign non-melanocytic lesions which can appear in the skin. The most common type are seborrhoeic keratoses (SKs, singular: seborrhoeic keratosis), which are proliferations of epidermal cells appearing especially among individuals over 40 years of age. SKs often appear as one or more, sharply defined, light brown, flat macules but can also present as elevated verrucous lesions with varying pigmentation (yellowish-brown, gray, pink, light to dark brown or black). Sometimes SKs can be difficult to differentiate from a MM.

Similarly, there are a wide range of malignant non-melanocytic lesions, of which BCCs and SCCs are by far the most frequently observed types. These cancers are commonly referred to as non-melanoma skin cancer (NMSC).

- **Basal cell carcinoma**

BCC is the most common type of skin cancer among fair-skinned individuals. BCCs are typically detected in the middle-aged and elderly population. These tumors grow slowly invading surrounding tissues but rarely metastasize. BCCs are thought to originate *de novo*,

i.e. without precursors, from immature cells of the lowermost cell layers of the epidermis.[20, 21] Histopathologically, various subtypes of BCC have been described (*e.g.* superficial, nodular, micronodular, cystic, pigmented, adenoid, infiltrating, sclerosing, keratotic, infundibulocystic, metatypical, basosquamous and fibroepitheliomatous). Mixed types are not uncommon.[21] Nodular BCCs appear most commonly in the head and neck region as elevated papules with a pearly appearance, superficial telangiectasias and, frequently, a central ulceration. Clinically, superficial BCCs present as pink to reddish-brown, flat or slightly elevated, scaly patches often located on the trunk. Infiltrating and sclerosing (morpheiform) BCCs are more aggressive, sometimes ulcerated, scar-like plaques or elevated lesions with ill-defined borders.

Microscopically, BCCs are composed of nests of basaloid cells with a haphazard arrangement of these cells within the center of the nests and palisading at the periphery. Numerous apoptotic tumor cells and mitotic figures can be found. Tumor nests are surrounded by a newly formed stroma which is different from the adjacent dermis. A variable inflammatory cell infiltrate is commonly present. Nodular BCCs consist of well-demarcated tumor nests and may present with ulceration in larger lesions. Retraction spaces can be seen between the nests and the surrounding stroma. Superficial BCCs are composed of numerous small nests of basaloid cells connected to the epidermis. Sclerosing BCCs are poorly demarcated lesions in which tumor cells grow as thin strands set in a dense fibrous stroma.

- **Squamous cell carcinoma and precursors**

SCCs are the second most common type of skin cancer and derive from atypical keratinocytes in the epidermis. These tumors arise most frequently on chronically UV-exposed areas such as the head and neck region and on the dorsum of the hands. Most SCCs are slow-growing lesions, which invade underlying structures having the potential to spread to regional lymph nodes and, less frequently, to distant organs. The metastatic potential increases in SCCs arising on the ear and lip; in SCCs on areas not exposed to UV

light (*e.g.*, the genital area); in immunosuppressed individuals such as organ transplant recipients (OTRs) and also in SCCs arising in chronically scarred or inflamed skin (*i.e.*, Marjolin's ulcer).[22, 23]

SCCs present clinically as indurated plaques or nodules with varying presence of ulceration and keratinous crusts. Actinic damage is frequently observed in surrounding skin. Histopathologically, nests of squamous epithelial cells arising in the epidermis extend into the dermis, accompanied by variable central keratinization and horn pearl formation. Depending on the degree of anaplasia in the tumor nests, SCCs are classified as well, moderately and poorly differentiated ('well differentiated' being the least aggressive form).[21]

AKs are the earliest precursors to SCC. They are almost exclusively found in the fair-skinned population and incidence increases with age as they are also due to chronic exposure to UV radiation. In Australia, AKs are present in 40-60 % of individuals over the age of 40.[24] Clinically, AKs typically have an erythematous base covered by scales (hyperkeratosis) ranging from discrete rough spots (usually 3-10 mm in diameter) on UV-exposed skin to elevated, hyperkeratotic plaques which can be several centimetres in diameter. Up to 10 % of all AKs can evolve into invasive SCCs after 10 years, but many remain unchanged and approximately 25 % regress spontaneously.[25-27] Most authors agree that their uncertain nature warrants treatment.[27] Histopathologically, AKs usually show focal parakeratosis, a loss of the underlying *stratum granulosum* and a slightly thickened epidermis with varying degrees of atypia. Different types of AKs exist, such as hyperplastic (hypertrophic), pigmented and lichenoid AKs.[21] The presence of AK on the vermilion part of the lip is known as actinic cheilitis.

Field cancerization is a term used by dermatologists to describe extensive areas of sun-damaged skin with multiple precancerous lesions (Fig. 4) and, at times, even invasive tumors. This is often present in elderly patients after a lifetime of chronic UV exposure or in OTRs. Field cancerization is challenging since early invasive SCCs are often indiscernible among all the precursors within these areas. Skin areas that are commonly affected include: the face, the scalp in balding men, the chest, the upper back and shoulders, the forearms, the dorsum of the hands and the lower legs.

Figure 4. Field cancerization of the forehead. Multiple AKs are present. (Photo: Morgan Carlsson)

The term SCC *in situ* or Bowen's disease is used when the atypical keratinocytes involve the full thickness of the epidermis without invading the dermis.[28] These lesions are characterized by a disorderly maturation of the epidermis, mitoses, multinucleate keratinocytes and dyskeratotic cells. Usually, parakeratosis and hyperkeratosis are present. Most SCC *in situ* present in fair-skinned older individuals as well-defined, erythematous, scaly plaques on sun-exposed areas. However, there are also genital lesions (Fig. 5) with the histopathology of

SCC *in situ* such as bowenoid papulosis, erythroplasia Queyrat (males) and severe vulvar intraepithelial neoplasia (females). The risk of progression to an invasive SCC is unclear but has been suggested to lie between 3-5 % for SCC *in situ* on sun-exposed skin and 5-10 % for erythroplasia Queyrat (SCC *in situ* of the glans penis).[29-31]
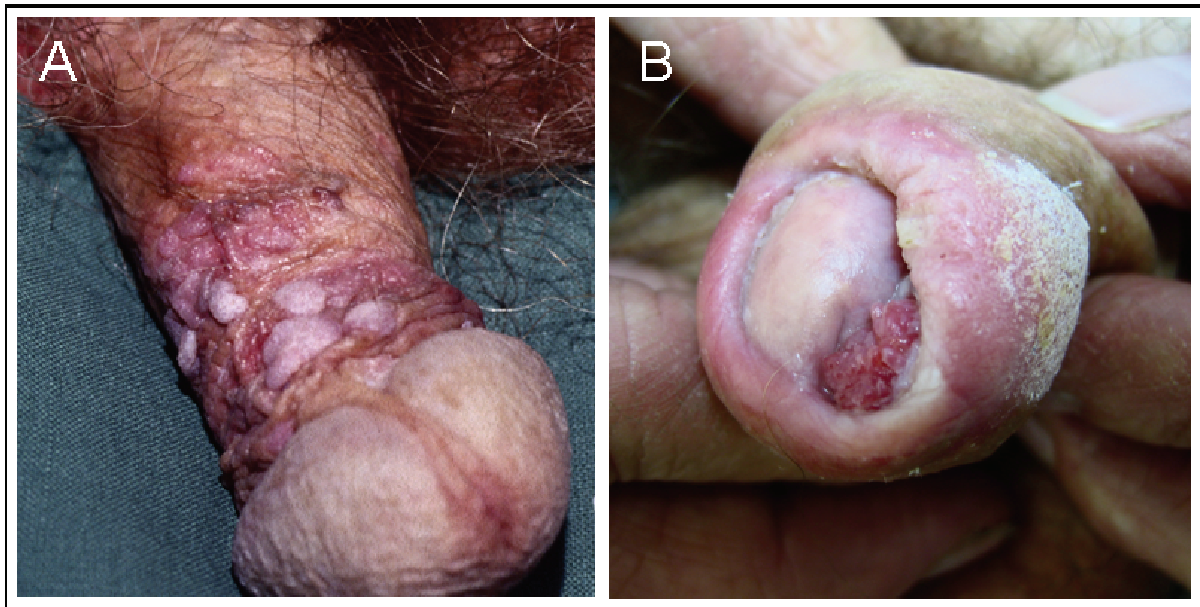
Figure 5. (A) PIN and (B) SCC of the penis. (Photo: John Paoli)

- **Penile intraepithelial neoplasia**

Penile intraepithelial neoplasia (PIN, Fig. 5) is a term used to describe varying degrees of intraepidermal cellular atypia in the male genital area of low, moderate or severe degree (PIN grades I-III). In PIN III lesions, histopathological signs of SCC *in situ* are observed and clinical presentations include Bowenoid papulosis, Bowen's disease and erythroplasia Queyrat.[32] PIN arising in middle-aged and elderly men has proven to be difficult to manage. There is a high risk of recurrence as well as a risk of progression to invasive SCC regardless of the treatment modality used.[33] An infection caused by high-risk human papilloma virus (HPV) is present in 70-100 % of all patients with PIN.[34] Penile SCC (Fig. 5) is also associated with the inflammatory skin disease lichen sclerosus and smoking. It is also more common in the population of men who are not circumcised as newborns.[34-38]

1.2.3 Etiology & risk factors

This section briefly summarizes the main risk factors associated with skin cancer development and also mentions some important high-risk patient groups.

- **Solar UV radiation**

The main cause of skin cancer development is exposure to solar UV radiation, a process known as photocarcinogenesis. This process involves an accumulation of genetic changes combined with a modulation of the immune system, which ultimately leads to the development of MM, SCC and BCC. Chronic UV exposure seems to be most important in the induction of SCCs, whereas it could be protective for the development of MMs.[39] Intermittent UV exposure seems to have a stronger correlation with the pathogenesis of MMs and BCCs.[40] UVB radiation, with a wavelength of 280-320 nm, can damage the DNA of both keratinocytes and melanocytes directly by inducing the formation of cyclobutane thymidine dimers in oncogenes and tumor suppressor genes.[41] On the other hand, UVA (320-400 nm) mainly does damage indirectly by inducing the production of free radicals and reactive oxygen species. To protect ourselves from this damaging radiation we produce melanin and also have active enzymatic DNA repair mechanisms (*e.g.*, nucleotide excision repair).[42]

- **Skin type and nevi**

In 1988, Fitzpatrick described a system to classify an individual's skin type based on skin color and susceptibility to UV radiation (how easily the individual tans and burns).[43] This classification is summarized in Table 2. Fair-coloured skin, light coloured or red hair and a poor ability to tan are phenotypic characteristics associated with the development of all main types of skin cancer.[44-46] Individuals presenting a large number of common and/or atypical nevi have been shown to have an increased risk of developing MM.[47] Atypical or dysplastic nevi are considered to be potential precursors of MM and markers of increased risk.[48] Patients born with large congenital melanocytic nevi with a projected adult size of > 40 cm

have an increased risk of developing MM, which often occurs at early ages with a fatal outcome.[49, 50]

Table 2. Sun-reactive skin type classification according to Fitzpatrick.

Skin type	Skin color	UV susceptibility (first exposure in summer)
I	White skin	Always burn, never tan
II	White skin	Usually burn, tan with difficulty
III	White skin	Sometimes mild burn, tan about average
IV	White skin	Rarely burn, tan with ease
V	Brown skin	Tan
VI	Black skin	Tan

- **Previous skin cancer**

A patient with a previous MM has a 9 times higher relative risk of developing a new MM compared to the normal population.[51] A new primary SCC is diagnosed in 30 % of patients within 5 years of a previous SCC and 52 % develop a new NMSC (SCC or BCC) within the same period of time.[52] Thirty-three percent of all patients diagnosed with a BCC develop a second BCC within 2 years.[53]

- **Dysplastic nevus syndrome**

Dysplastic nevus syndrome (DNS) is an autosomal dominant hereditary condition with incomplete penetrance. However, gene mutations (*e.g.* mutations in the CDKN2A gene) are found in a low number of DNS patients, which suggests that unknown genes may also be involved in the development of DNS.[54] Patients with DNS typically present a phenotype with a variable amount of atypical or dysplastic nevi (Fig. 6) associated with familial history of MM.[55, 56] Patients with DNS are considered a high-risk group since their risk of developing MM has been estimated to be 85 times higher than the normal population in those with dysplastic nevi and 229 times higher if the patient has had a previous MM.[57]

- **Immunosuppression in OTRs**

The immunosuppressive drugs given to OTRs in order to prevent organ rejection unfortunately have the side effect of an approximately 100-fold increased risk of developing SCC (Fig. 6), a 6-10 fold increased risk for BCC and possibly a slightly increased risk of developing MM.[58-61] Therefore, OTRs should be considered high-risk patients and require close follow-up in order to diagnose and treat these lesions at an early stage.
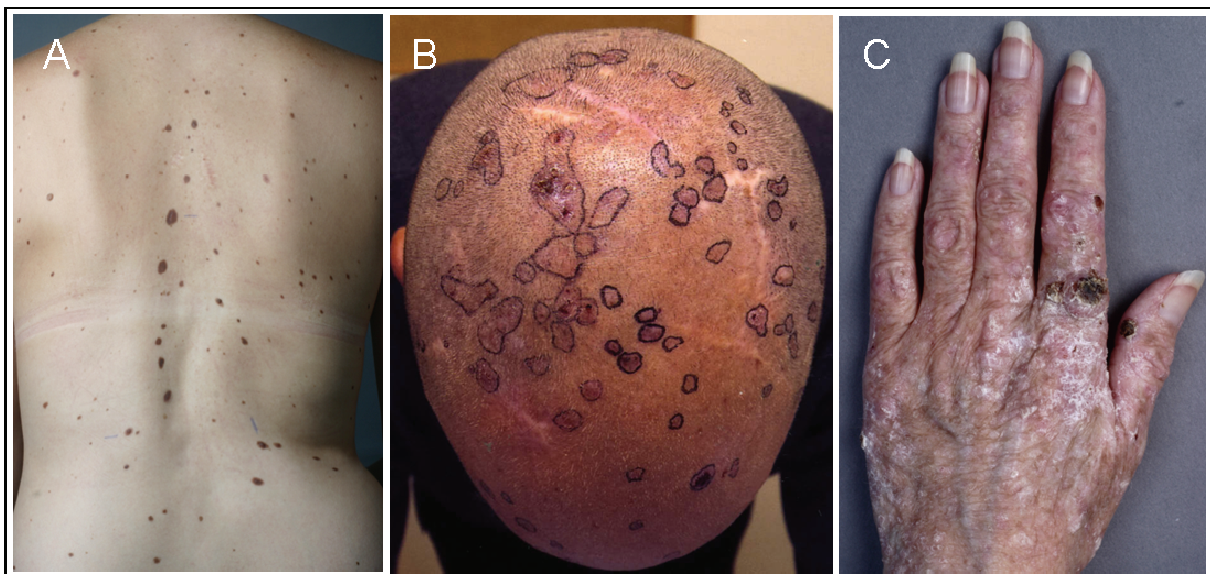
Figure 6. Examples of high-risk patients: (A) DNS patient with multiple atypical nevi, (B) Gorlin's syndrome with several BCCs on the scalp and (C) an OTR with field cancerization of the dorsum of the hand and numerous invasive SCCs. (Photo: Morgan Carlsson and Karin Terstappen)

- **Genetic disorders**

The Gorlin-Goltz syndrome is a rare genetic disease inherited in an autosomal dominant manner and characterized by a an extraordinary predisposition to developing multiple BCCs (Fig. 6) in association with a series of malformations and/or anormalities (*e.g.*, odontogenic keratocysts of the jaw, bifid or fused ribs, calcification of the falx cerebri, macrocephaly, palmar or plantar pits and medulloblastoma during childhood).[62]

Another rare genetic disorder associated with a highly increased risk of MM, SCC and BCC is xeroderma pigmentosum.[63] Patients with this disease are unable to repair UV-induced DNA damage in their skin cells making them highly susceptible to all types of skin cancer.

Oculocutaneous albinism; a group of inherited disorders of melanin biosynthesis in which patients present with general reduction in hair, skin and eye pigmentation; also increases the risk of developing skin cancer.[64]

Epidermodysplasia verruciformis is an extremely rare autosomal recessive genetic disease characterized by an abnormal susceptibility to HPV infections of the skin and a high risk of SCC.[65, 66] Several HPV types are detected in patients with epidermodysplasia verruciformis (*e.g.* 5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 50), but HPV types 5 and 8 seem to have a stronger association with the development of SCCs (see section on HPV below).[66]

Recessive dystrophic epidermolysis bullosa is characterized by repeated blister formation. The patients who survive recurrent bacterial sepsis during infancy have a 50-fold increased risk of developing SCCs in chronic non-healing wounds.[67]

- **HPV**

Genital SCCs and PIN are often associated with HPV infection as mentioned earlier. There are several well-known mucosal high-risk HPV types belonging to the genus *Alphapapillomavirus* (*e.g.* 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68 and 69), which are regularly associated with *in situ* lesions or invasive carcinomas of the cervix, vagina, vulva, penis and anus.[68] HPV 16 and 18 are by far the most common types in such neoplasias. Vaccines for HPV infections have recently been introduced in young female individuals in an attempt to prevent cervical cancer (HPV types 16 and 18) and genital warts

(HPV types 6 and 11).[69] Although the vaccines are still not available for male individuals, this preventive measure may even reduce the incidence of PIN and other mucosal cancers in the future.

Cutaneous HPV types belonging to the genus *Betapapillomavirus* can be found in normal skin, in benign skin lesions and in NMSCs, especially cutaneous SCCs.[70, 71] HPV DNA, most commonly from HPV 5 and 8, can be detected in approximately 75 % of all SCCs in OTRs.[71] In immunocompetent patients, the prevalence of cutaneous HPV types is increased at sun-exposed skin sites, which may result from an amplification of the viral genome caused directly by UV light or from UV-induced local immunosuppression.[72] It has been suggested that cutaneous HPV types may contribute to carcinogenesis, but the mechanisms behind this are unknown. HPV produces oncoproteins (*e.g.* E6 and E7), which can inhibit UVB-induced apoptosis. This could subsequently lead to the propagation of harmful UV-induced mutations.[73]

- **Other risk factors**

A very small proportion of NMSCs occur due to previous ionising radiation therapy, arsenic or chronic ulcers, sinus tracts and scars.[44]

1.3 Diagnostic methods

Diagnosing skin cancer, especially MM, at an early stage is essential to achieve reduced mortality and morbidity. Today, most dermatologists and other physicians use a combination of visual examination, dermoscopy and histopathology to diagnose skin cancer. Several imaging techniques presented here are under development and may become valuable complements to today's mainstream diagnostic methods.

1.3.1 Visual examination

Well-trained dermatologists can diagnose MMs and NMSCs with high sensitivity (93.3 %) and specificity (97.8 %) using the naked eye, but the positive predictive value (suspected lesions that were true cancers) can be as low as 54 %.[74] In screening campaigns carried out in the United States in which only visual examination was used, only 2 out of 10 suspected MMs were confirmed.[75] Thus, a lot of suspicious lesions are removed unnecessarily. A Swedish study showed that 34 % of 174 confirmed MMs were not clinically suspected by the dermatologist.[76] However, the diagnostic accuracy for MM has been shown to be higher if the physician has over 10 years experience and with exposure to more than ten cases per year.[76, 77] Furthermore, general practitioners in the United Kingdom failed to recognize one-third of 36 skin malignancies in another study.[78]
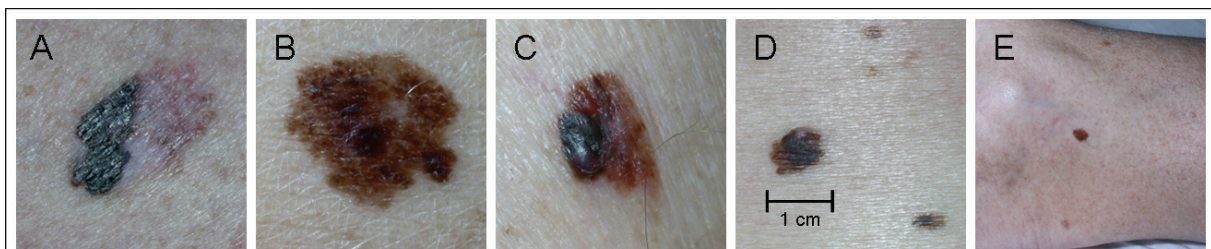
Figure 7. The "ABCDE" criteria may help differentiate MMs from benign nevi. The ABCDE acronym stands for: (A) **A**symmetry, (B) irregular **B**orders, (C) **C**olor variation, (D) a **D**iameter >6 mm and (E) a history of change or **E**volution. In the last case (E), the ABCD criteria are not fulfilled but the lesion has grown the past month and this proved to be a thin MM. (Photo: John Paoli and Morgan Carlsson)

Physicians recommend patients to perform skin self-examination regularly in order to detect skin cancer at an early stage.[79] Most patients with skin cancer have no other symptoms other than the visual presence of the lesion. Almost 60 % of all primary MMs and almost 75 % of all MM recurrences are detected by patients themselves.[80-82] In this sense, mnemonics such as the "ABCDE" criteria[83, 84] (Fig. 7) or the "ugly duckling sign"[85] (Fig. 8) can be useful for both patients and physicians in the early detection of MM.
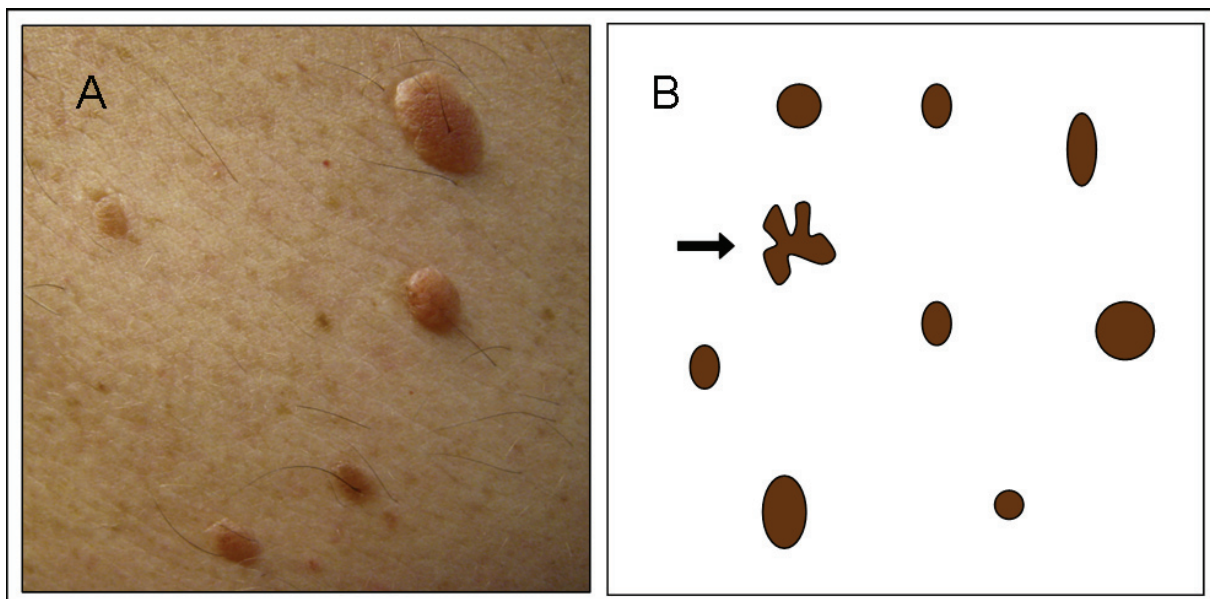
Figure 8. (A) Nevi in an individual tend to resemble each other. (B) The "ugly duckling sign" is based on the proposition that if a nevus stands out and looks different compared to the others (arrow), this lesion should be considered suspicious. (Photo and artwork: John Paoli)

1.3.2 Biopsy and histopathology

The gold standard in establishing the diagnosis of skin cancer is histopathology, *i.e.* the study of microscopic anatomical changes in abnormal tissue.[86, 87] A biopsy is the removal of a tissue sample for further histopathological examination. Incisional biopsies, in which only part of the lesion is sampled, are normally performed on NMSCs. Incisional biopsies can be performed with a scalpel resulting in a biopsy with an elliptical shape or by punch biopsy using a round shaped knife with a diameter of 2-8 mm (Fig. 9). Excisional biopsies, in which

the entire lesion is removed, are the most common technique for melanocytic lesions. Other techniques used to acquire tissue samples from skin lesions are shave biopsies, curettage biopsies, saucerization biopsies and fine needle aspiration biopsies. Tissue samples are processed by fixation, dehydration and infiltration, embedding, sectioning and staining before the histological slides are examined under a microscope by a pathologist.
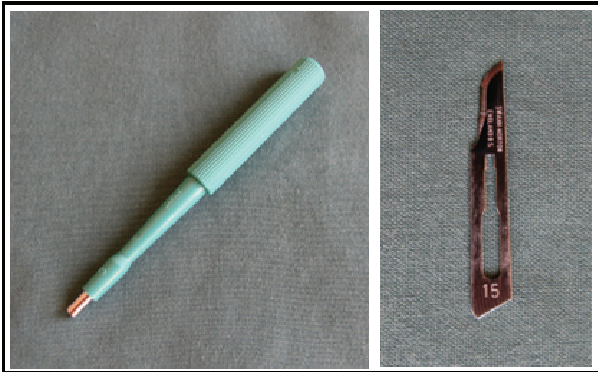
Figure 9. Punch biosy (left) and scalpel blade (right). (Photo: John Paoli)

In routine histopathology of skin cancer, the tissue samples are vertically sectioned. Histopathological examination also provides information on whether or not the lesion has been removed completely or not. The disadvantage of the vertical sections is the fact that less than 1 % of the excised area is studied, which can result in false reports of complete excisions (Fig. 10).[88] In contrast, the horizontal sections obtained during Mohs micrographic surgery (see section 1.4.1) observe 100 % of the lesion's margins.
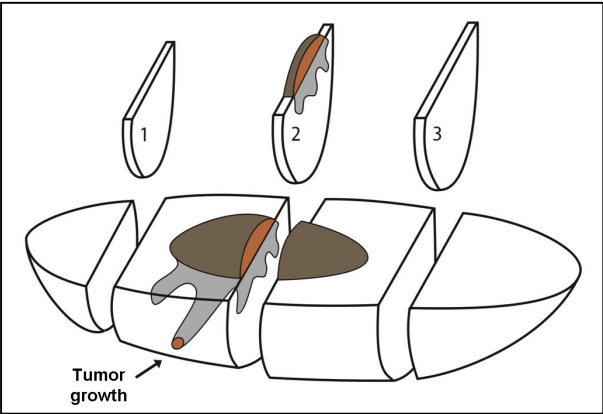
Figure 10. The disadvantage of vertical "bread-loaf" sectioning of excisional biopsies is that a tumor may be incorrectly determined to have clear margins due to missed finger-like extensions of tumor in the unexamined intervals (arrow). (Artwork: John Paoli)

1.3.3 Sentinel lymph node biopsy

The sentinel lymph node is, hypothetically, the first lymph node or group of nodes reached by lymphatic spread of cancer cells from a tumor. A sentinel lymph node biopsy is recommended for patients with MMs with a Breslow thickness >1 mm and in thinner MMs if they present with a Clark level of IV-V or with ulceration.[86] Preoperatively, a radioactive substance is injected around the tumor area and a lymphoscintigraphy is performed to map the tumor's lymphatic drainage (*e.g.* the axillary lymph nodes in a MM of the upper extremity). About 15 minutes before the actual biopsy, the physician injects a blue dye in the same area. Subsequently, the skin is incised over the area of interest. Through visual inspection of the stained lymph node(s) and using a Geiger counter to assess uptake of the radioactive substance, one or several nodes are removed for histopathological examination. If the sentinel lymph node(s) is positive, regional lymphadenectomy is considered. The sentinel lymph node biopsy technique is used in the staging of MM, as the detection of lymph node involvement is helpful in establishing stratification criteria for trials on adjuvant therapy. In patients without clinical evidence of nodal involvement, sentinel lymph node status is the most important prognostic factor, followed by tumor thickness and ulceration.[89]

1.3.4 Imaging techniques

Recently, several non-invasive diagnostic procedures based on optical imaging have been introduced in order to facilitate the diagnosis of skin cancers.

- **Dermoscopy**

Dermoscopy is the most widely used non-invasive diagnostic technique in clinical practice. Dermoscopy uses magnifying objectives and a transilluminating light source to visualize subsurface anatomic structures of the epidermis and papillary dermis as well as subtle clinical patterns of skin lesions.

Figure 11. Examples of devices used for dermoscopy: (A) contact dermoscopy, (B) non-contact dermoscopy with polarized light, (C) portable digital dermoscopy and (D) digital dermoscopy for total body photography of high-risk patients. (Photo: John Paoli)

There are several algorithms for the diagnosis of melanocytic lesions (*e.g.*, pattern analysis, ABCD-rule, Menzie's method and 7-point checklist) which, in experienced hands, increase the diagnostic accuracy for melanoma.[90] The diagnosis of non-melanoma skin cancer can also be enhanced with dermoscopy.[87] In high-risk patients with multiple atypical nevi, total body photography and digital dermoscopy allow dermatologists to detect MMs at early stages as discrete changes in these nevi can be observed at follow-up.[91] Different types of dermoscopes and a comparison of visual examination with dermoscopy are illustrated in Figures 11 and 12, respectively.
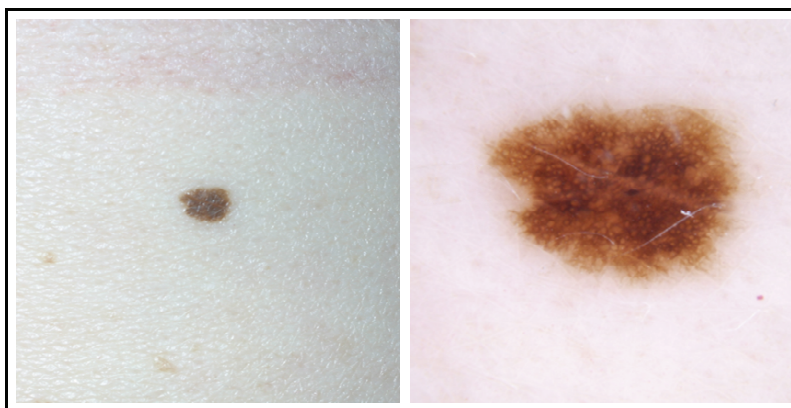
Figure 12. A benign nevus as viewed with the naked eye (left) and with dermoscopy (right). With dermoscopy a typical reticular pigment network can be observed correlating with the histopathological presence of melanin at the dermo-epidermal junction. (Photo: John Paoli)

- **Spectrophotometric intracutaneous analysis**

Spectrophotometric intracutaneous analysis is a non-invasive scanning technique in which a lesion is illuminated with visible and infrared light (400-1000 nm) that interacts with skin

tissue cromophores. The wavelength-dependent absorption and scattering characteristics of these cromophores allow for an *in vivo* analysis, which generates images of epidermal and dermal melanin, as well as the collagen and vasculature within the papillary dermis.[92] This diagnostic method has, unfortunately, not shown any advantage over dermoscopy in the diagnosis of MM or pigmented BCCs nor can it give reliable guidance for localizing the maximum tumor thickness of MMs prior to histopathological examination.[93-95] A system for spectrophotometric intracutaneous analysis is commercially available (SIAscope, Astron Clinica, Cambridge, U.K.).[92]

- **Fluorescence diagnosis**

Fluorscence diagnosis is a digital imaging technique used to detect skin tumor margins through registration of fluorescent light emitted from fluorophores in the skin after excitation of these molecules with light sources of specific wavelengths. These fluorophores can be endogenous (autofluorescence) or porphyrins produced after topical application of photosensitizer prodrugs such as 5-aminolevulinic acid (ALA) or its methyl ester, methyl aminolevulinate (MAL). Fluorescence diagnosis is a method under development, which could potentially demarcate tumor margins preoperatively. This could minimize the risk of incomplete excisions and reduce the number of excisions during staged tumor removal.[96]

- **Optical coherence tomography**

In optical coherence tomography (OCT), cross-sectional images of skin (resembling ultrasound images) are obtained by measuring the reflection of infrared radiation at a wavelength of 1300 nm scanned across the tissue. Skin cancer diagnosis has proven to be difficult with OCT as the technique cannot discern cell morphology (axial resolution = 8-10 µm, lateral resolution = 20-24 µm). However, the imaging depth in skin of approximately 2 mm could potentially help in determining tumor thickness.[97] Further studies are needed before OCT can be included in clinical practice.

- **Reflectance-mode confocal laser scanning microscopy**

Morphologic changes on the cellular level can be visualized non-invasively with reflectance-mode confocal laser scanning microscopy (RCLSM). This technique uses near-infrared (NIR) light to acquire horizontal optical sectioning of the skin down to the papillary dermis (approximately 200 µm). In RCLSM, only the reflected light from the focal point is detected since the rest of the backscattering is filtered out by a pinhole. Cellular structures can be imaged as the resolution is approximately 0.5-1.0 µm in the lateral direction and 3–5 µm in the axial direction. Promising results have been published on the use of RCLSM for skin cancer diagnosis and instruments for clinical use are commercially available (Vivascope 1000 and Vivascope 1500, Lucid Inc., Henrietta, New York, USA).[98-100] Recent studies have shown that the sensitivity, specificity, positive predictive value and the negative predictive value of this method are similar to those of dermoscopy.[99, 101] Some specialized skin cancer centers have started to use RCLSM in a clinical setting.

- **Multiphoton laser scanning microscopy**

Multiphoton laser scanning microscopy (MPLSM) is a laser scanning imaging technique that, similar to RCLSM, also entails horizontal optical sectioning of biological tissue.[102] However, instead of recording the backscattered light, as in RCLSM, MPLSM involves the use of non-linear optical processes. Most commonly, the process of two-photon excitation (2PE) is applied for visualization of fluorescent substances. The difference between conventional one-photon excitation (1PE) and 2PE is illustrated schematically in Figure 13.

As illustrated by the figure, 2PE involves near-simultaneous (within 10^{-18} s) absorption of two low-energy photons by a fluorescent molecule, which results in the emission of a fluorescence photon, typically of higher energy than the two excitatory photons. As the probability of 2PE occurring is low, a high flux of excitation photons is required, which generally is obtained from a femtosecond pulsed laser. The use of NIR excitation light enables deeper light penetration in biological tissue, compared with 1PE applied in

conventional fluorescence microscopy using UV or visible light. Furthermore, excitation with NIR light is more appropriate for *in vivo* imaging of biological tissues as it is less harmful than high-energy UV excitation wavelengths. The non-linearity of the excitation process means that 2PE will only occur at the focal point. Thus, fluorescence from a thin optical section can be detected without having to filter out backscattered light with a pinhole as in RCLSM.
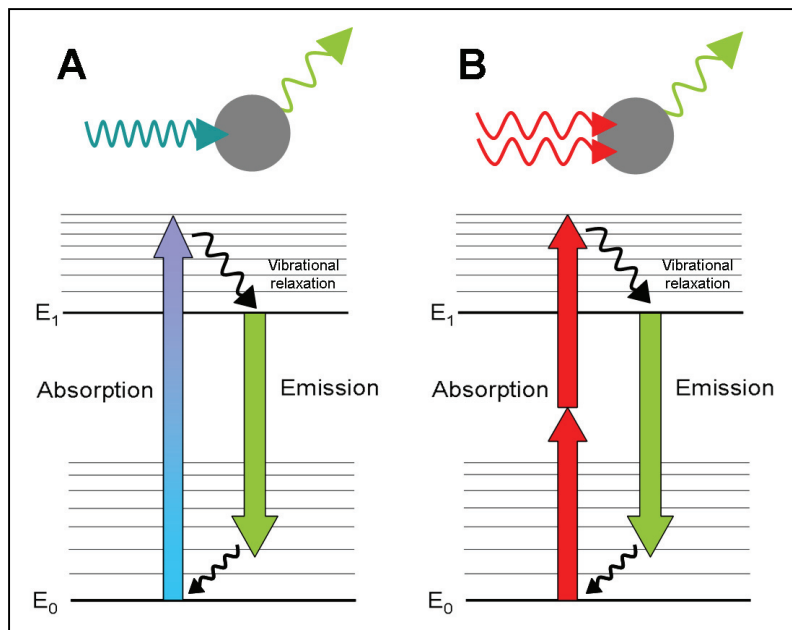
Figure 13. Absorbtion of light by a fluorescent molecule (gray circle) leads to an energy transition from the molecular ground state, E_0, to a higher electronic energy state, E_1. (A) In 1PE, the fluorescent molecule absorbs a high-energy photon with a short wavelength (blue arrows), which triggers the emission of a fluorescent photon with less energy and a longer wavelength (green arrows). (B) In 2PE, two low-energy photons of a longer wavelength (red arrows) are absorbed simultaneously, leading to emitted light of the same energy as with 1PE. (Artwork: Marica B. Ericson and John Paoli)

When imaging human skin with MPLSM, NIR light is used to excite fluorophores, which normally are excited by UV or visible light. These fluorophores can either be endogenous autofluorescent substances (*e.g.* reduced nicotinamide adenine dinucleotide [NADH], keratin, melanin, collagen and elastin)[103, 104] or exogenous fluorescent markers added to the skin specimen[103, 105]. Since endogenous fluorophores can be visualized, the technique has the potential of becoming a bedside non-invasive imaging technique with cellular and subcellular resolution. By scanning parallel to the skin surface, two-dimensional images (*i.e.* in the *x-y* plane) are obtained, as illustrated by Figure 14. By sequentially varying the scanning depths (*z*-levels), three-dimensional optical sectioning of the tissue can be achieved.
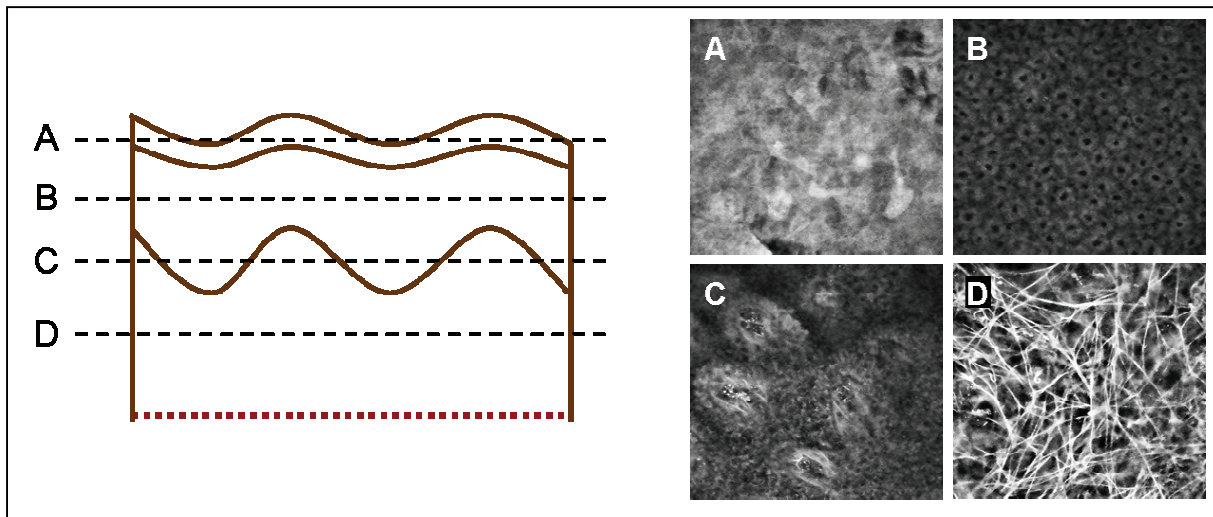
Figure 14. Illustration of optical sectioning of healthy human skin performed using MPLSM (left) and examples of images (right) obtained from different tissue layers. (A) Sections through the *stratum corneum* show large scale-like cells with highly fluorescent keratin. (B) Regularly distributed keratinocytes are visualized within the *stratum spinosum*. Cytoplasmic NADH is the main source of fluorescence. (C) The rounded dermal papillae containing dermal fibers are observed among the multitudinous basal cells, which fluoresce due to their high melanin content. (D) A complex network of highly fluorescent collagen and elastin fibers are seen within the papillary dermis. All MPLSM images are 323 x 323 μm. (Photo and artwork: John Paoli)

MPLSM has been used *in vivo* and *ex vivo* for the study of human skin[106], but there are few studies concerning the diagnosis of NMSC[107, 108]. Paper I was the first study on MPLSM which investigated NMSC without previous sectioning of the skin.[108] Recently, a large study on benign and malignant melanocytic lesions was also published.[109] A commercially available MPLSM system (*DermaInspect*®, JenLab, Germany) has been approved for clinical use[109, 110], but further development is necessary before the technique gains clinical acceptance, as will be discussed in "Outlook for the future" (see section 7).

1.3.5 HPV detection

As mentioned earlier, HPV is often associated with SCC in patients with PIN lesions and in OTRs. HPV detection is not regularly performed to establish the precise etiology of common lesions, but this can be carried out in research scenarios. Tissue samples are collected from the lesion through biopsies or swab sampling. A swab is a small piece of absorbent material

attached to a stick, which can be rubbed against the skin to acquire tissue samples. A polymerase chain reaction, a technique widely used in molecular biology, is used to detect HPV DNA. Subsequently, a technique known as reverse hybridization is carried out to confirm the HPV type (*e.g.* HPV 16).[111] HPV detection using these methods was performed in Paper II.

1.4 Treatment

Skin cancer can be treated by several different methods: surgery, destructive therapies, medical treatments and radiotherapy. Surgery is the gold standard of treatment for primary MMs, SCCs and most BCCs. However, other methods can be applied for effective therapy of SCC precursors and nonaggressive BCCs. The main indications and the effectiveness of the different treatment methods are summarized in Table 3.

Table 3. Effectiveness of different therapeutic modalities for different types of NMSC in general terms. Variations in treatment results due to special characteristics of the lesions and/or particular body sites are not considered here.[25, 27, 29, 44, 112-115]

Lesion type	Surgery	MMS	C&E	Cryo	Laser	PDT	Imiq.	5-FU	Rx
Single AK	N.A.	N.A.	N.A.	+++	+	N.A.	N.A.	N.A.	N.A.
Multiple AKs	N.A.	N.A.	N.A.	++	+	+++	+++	+++	N.A.
Small SCC*is*	++	(+++)	+++	+++	+	+++	N.A.	++	(+++)
Large SCC*is*	++	(+++)	+	++	-	+++	N.A.	++	(+++)
Low-risk SCC	+++	(+++)	(++)	(++)	N.A.	N.A.	N.A.	N.A.	(+++)
High-risk SCC	+++	+++	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	++
sBCC	+++	(+++)	+++	+++	+	+++	++	++	(++)
nBCC	+++	(+++)	+++	+++	-	+	N.A.	N.A.	(+++)
iBCC	+++	+++	-	+	N.A.	N.A.	N.A.	N.A.	(+++)
aBCC/rBCC	++	+++	-	-	N.A.	N.A.	N.A.	N.A.	(++)

Cryo, cryosurgery; Imiq, imiquimod, Rx, radiotherapy; SCC*is*, SCC *in situ*; sBCC, superficial BCC; nBCC, nodular BCC; iBCC, infiltrating BCC; aBCC, aggressive or sclerosing; rBCC, recurrent BCC; +++, recommended; ++, effective; +, relatively effective; -, not recommended; N.A., not applicable or generally not used; (++/+++), effective but unnecessary or not first-line therapy.

1.4.1 Surgery

Surgical excision implies the removal of an entire lesion along with a border (margin) of clinically healthy skin using a scalpel (sharp knife). Surgery of skin cancer is usually performed with an elliptical or fusiform excision. The ellipse is commonly designed with a length 3 times the width and the major axis directed so that the subsequent scar runs within or parallel to existing relaxed skin tension lines. The defect that results from the excision is

closed by bringing the wound edges together with appropriate sutures. Several guidelines stating the recommended excision margins for MMs, SCCs and BCCs have been published.[86, 115, 116] Following excision, the tissue samples undergo histopathological examination with vertical ("bread-loaf") sectioning as mentioned earlier. The clear advantage of surgery with respect to other treatment alternatives is the possibility of histopathological verification of the diagnosis and the complete removal of the tumor.[44, 112]

- **Mohs micrographic surgery**

Mohs micrographic surgery (MMS) is a more advanced surgical technique based on horizontally orientated tissue sections for histopathologically controlled, staged tumor removal. The original "chemosurgical" fixed-tissue technique with zinc chloride described by Fredric E. Mohs in 1941 has developed through the years and, nowadays, MMS is carried out with a fresh-tissue technique, which allows for tumor removal in a single day in most cases.[117, 118] In MMS, all of the lesion's margins are histopathologically examined after each surgical stage. This is made possible through saucer-like excisions of the lesion with a 45° angle at the lateral margins, which can be flattened into the same plane as the deep margins prior to sectioning. Small markings in the removed specimen and the remaining wound are made to create a map, which allows for any residual tumor to be removed selectively in subsequent surgical stages. This ensures complete removal of the tumor while sparing as much healthy tissue as possible. MMS can be applied on a long list of skin cancers, but is mainly used for the removal of locally aggressive tumors which are difficult to eradicate with routine surgery; tumors located in areas where tissue preservation is important or recurrent tumors.[117, 119] When abiding by these indications, MMS centers in Europe achieve 5-year recurrence rates of approximately 3 % for primary BCCs and 7 % for recurrent BCCs.[120, 121] In Sweden, MMS is only performed at Sahlgrenska University Hospital in Gothenburg on aggressive or recurrent BCCs in the facial area.[119]

1.4.2 Destructive methods

Well-defined and nonaggressive primary NMSCs confined to the epidermis and dermis can in many cases be treated effectively with destructive methods such as curettage and electrocautery or electrodesiccation (C&E), cryosurgery (with or without previous curettage) and ablative laser surgery. Destructive methods are rapidly performed under local anesthesia and are very cost-effective. However, these modalities require a previous biopsy to confirm the histopathological diagnosis since they destroy all evidence of the tumor's existence. Furthermore, these treatments require an adequate technique by the physician in order to be effective. Another disadvantage is the fact that the resulting wounds have to heal by secondary intention, which can lead to scarring, hyper- or hypopigmentation.[112]

Figure 15. Materials used for (A) curettage, (B) electrocautery and (C) cryotherapy. (Photo: John Paoli)

- **Curettage and electrocautery or electrodesiccation**

C&E involves mechanical removal of friable tumor tissue from the firm, healthy tissue using a curette with a semi-sharp edge (Fig. 15) followed by thermal destruction of the microscopic remains. The resulting eschar (or scab) is commonly curetted and treated with electrocautery or electrodesiccation at least one more time before letting the wound heal by secondary intention.[112] C&E is generally recommended for small BCCs on low-risk body sites such as the trunk or the extremities. Lesions larger than 6 mm in the facial area have 5-year recurrence rates of approximately 20 % with C&E.[122]

- **Cryosurgery**

Cryosurgery uses liquid nitrogen (-196°C) administered using spray guns (Fig. 15) to destroy a tumor. Epidermal lesions can often be treated with one or two freeze-thaw cycles alone, but hyperkeratotic and thicker lesions generally require previous curettage of the tumor. Cure rates for cryosurgery on BCCs are in general above 90 %.[123] This method has also been shown to be an excellent alternative to surgery on non-aggressive BCCs of the ear, nose and eyelids.[124-126] As mentioned earlier, the use of a proper technique will clearly affect the response rates. In one study, complete response rates of 39 % were obtained with 1-5 s freeze times on AKs compared to 80 % response rates using 10-15 s freeze times.[127] Certain locations should be considered relative contraindications for cryosurgery (*e.g.* the eyebrows and the hairy scalp, due to the risk for scarring alopecia; and the lower legs, due to the risk for ulceration).[128] SCC *in situ* lesions of the lower legs treated with cryosurgery take a longer time to heal when compared to C&E.[127] The most common adverse event of cryosurgery is hypopigmentation while scarring is uncommon.[129]

- **Laser ablation**

Another destructive modality which may be effective on superficial BCCs, AKs and SCC *in situ* lesions is vaporization by ablative laser surgery with carbon dioxide (CO_2) or erbium:YAG lasers.[44] CO_2 laser ablation is often used on genital lesions such as PIN.[29] In general, this technique is still an uncommon form of treatment compared to C&E or cryosurgery and little follow-up data has been published.[116] Laser ablation requires careful selection of the treated lesions and experience with the technique.[130]

1.4.3 Medical treatments

The most important medical treatments for NMSCs are summarized below. Superficial NMSCs can be treated with several topical drugs and photodynamic therapy (PDT).

Retinoids can be used for the chemoprevention of SCCs and other systemic treatments are tested for advanced disease.

- **Topical drugs**

Topical drugs such as diclofenac, 5-fluorouracil (5-FU) and imiquimod can be used to treat certain superficial NMSCs and so-called field cancerization. These medical modalities all have in common the need for several weeks of application in order to be effective and the adverse effects of erythema, edema, scaling, erosions and pruritus in the treatment area (Fig. 16). Imiquimod can also cause fever, especially when larger areas are treated. Lack of patient compliance can become an issue with topical drugs due to the long duration of treatment and the adverse effects.

Figure 16. Severe adverse reaction with erythema and thick crusts after a 4-week period with imiquimod for extensive AKs on the cheek. (Informed consent obtained, photo: John Paoli)

Topical diclofenac in hyaluronic acid can be used in the treatment of AKs but daily treatment during 90 days is required, which increases the risk of poor compliance. Diclofenac inhibits prostaglandin E2 synthesis, thus eliminating the suppression of the production of immune-regulatory lymphocytes, T- and B-cell proliferation and the cytotoxic activity of natural killer

cells. Diclofenac also inhibits cyclo-oxygenase 2, which can decrease tumor angiogenesis. Complete resolution of AKs has been estimated to be 50 %.[131] Meanwhile, clearance of at least 75 % of the target lesions has been observed in approximately 70 % of patients completing the 90-day treatment period.[132]

5-FU is a topical chemotherapeutic antimetabolite which interferes with the synthesis of DNA and, to a lesser extent, inhibits the formation of RNA. 5-FU is applied twice daily during 2-4 weeks as a 5% cream and can be used for the treatment of AK, SCC *in situ* and superficial BCC. Currently, 5-FU is not commercially available in Sweden. Cure rates of 90 % have been observed when treating small superficial BCCs.[133] In patients with multiple AKs, several open trials show that 5-FU can achieve clearance of more than 70 % of the lesions.[25] In a randomized controlled trial, 5-FU was as effective as PDT in the treatment of AKs on the dorsum of the hands.[134] However, PDT has been shown to be more effective and cause less adverse effects than 5-FU in the treatment of SCC *in situ*.[135]

Imiquimod is an immune-response modifier with antiviral and antitumor activity. These effects are achieved as imiquimod acts through toll-like receptors to induce cytokine and chemokine production promoting both innate and adaptive cell-mediated immune responses. Imiquimod is approved by the European Medicines Agency for the treatment of AKs and superficial BCCs.[116, 136] It is labelled for superficial BCCs in Europe, Australia and the USA.[27] In addition, relatively promising results have also been shown in the treatment of SCC *in situ* and even lentigo maligna.[29, 137] The recommended treatment protocol for imiquimod 5% cream when treating primary small superficial BCCs is five times per week for 6 weeks.[116] Using this regimen, 80 % of small superficial BCCs present histopathological clearance.[138] AKs are treated once daily, 3 days per week, during 4 weeks. The effect of this therapy is evaluated after follow-up at 4 weeks and a new treatment course of 4 weeks is carried out when necessary.[136] Four out of five patients show a 75 % reduction in the number of AKs with this method.

- **Photodynamic therapy**

PDT is another medical treatment for superficial NMSCs and field cancerization. This method is based on a two-step process (Fig. 17). First, a prodrug such as ALA or MAL is topically applied to the lesion(s) and the treatment area is covered with an occlusive dressing for 3 hours. During this time, ALA or MAL will enter the heme biosynthesis pathway resulting in the accumulation of the endogenous photosensitizer protoporphyrin IX (PpIX), preferentially within tumor cells.[139] The dressings and excess cream are removed prior to illumination of the area with red light, which is the second stage of PDT. Illumination can be performed with various light sources including light emitting diodes, lasers, fluorescent lamps and broadband light sources. In Europe, most physicians use the red 630 nm absorption peak of PpIX with deep penetration in the skin, whereas the blue 410 nm absorption peak with less penetration is more commonly utilized in the USA.[140] The light interacts with PpIX causing reactive oxygen species (especially singlet oxygen) to be produced, which ultimately leads to tumor destruction.[141] PDT has a significantly better cosmetic outcome than other conventional therapies when treating AK, SCC *in situ* and BCC.[141] The indications and effectiveness of PDT are summarized in Table 3. The main drawback of PDT is the pain experienced during the illumination phase.[142, 143]

The mechanisms involved in pain during PDT have not been fully elucidated. Most patients perceive a painful burning or stinging sensation in the treated area[142], which is greatest during the first minutes of irradiation and decreases towards the end of this phase[144]. Patients with large areas of multiple AKs on the face and/or scalp have been found to have more pain than others.[142, 143] In some cases, the intensity of the pain is unbearable, forcing treatment to be interrupted.[145] For years, all strategies which have been applied to reduce pain have been minimally effective or completely unsuccessful (*e.g.* cooling with air or water, topical anesthetics, premedication with oral analgesics, fractionated treatment and transcutaneous electrical nerve stimulation).[146-151] Effective analgesia can be accomplished with intradermal injections of local anesthetics or subcutaneous infiltration anesthesia[152], but

these strategies are not possible when the whole forehead is involved for example. Paper II and, especially, Paper III report on the effectiveness of nerve blocks in relieving pain during PDT.[153, 154]
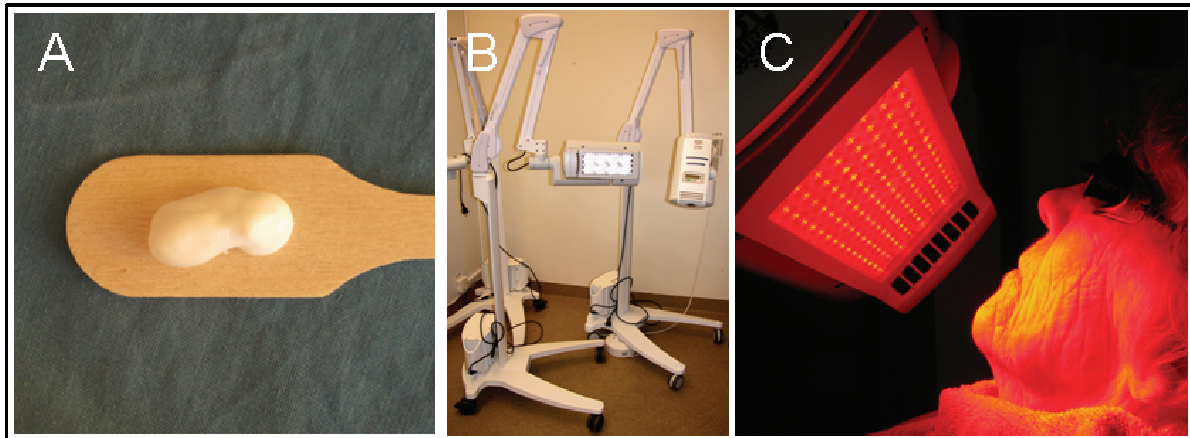
Figure 17. PDT is a two-step procedure in which (A) a cream containing a photosensitizing agent is applied to the treatment area prior to (B and C) illumination with specific light sources. (Photo: John Paoli)

Nerve blocks

Peripheral nerve blocks are well-known anesthetic techniques used to provide regional anesthesia. This is accomplished through local injection of an anesthetic solution (*e.g.* lidocaine, bupivacaine) in the proximity of the appropriate nerve interrupting the sensory nerve conductivity of that region of the body. The technique is routinely used within dentistry, during head and neck surgery and in surgical procedures of the hand.[155-158] Nerve blocks use small amounts of anesthetic solution and can be performed in ambulatory settings.[157] In Paper II, dorsal penile nerve blocks (Fig. 18) are employed to relieve pain during PDT for PIN lesions. Meanwhile, Paper III presents the novel use of supraorbital/supratrochlear, infraorbital and mental nerve blocks (Paper III, Fig. 1) in pain relief during PDT for extensive AKs in the facial area.
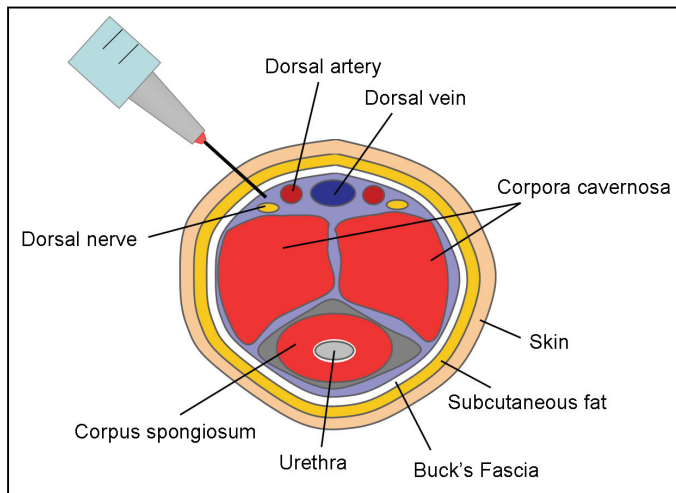
Figure 18. Cross section of the base of the penis. The injection site for the dorsal penile nerve block in the proximity of the dorsal nerve is shown. Bilateral injections are performed. (Artwork: John Paoli)

- **Retinoids**

Retinoids are vitamin A derivatives which can be used in the chemoprevention of skin cancer by inducing growth arrest or apoptosis of tumor cells and/or by modulating the immune response or the differentiation of keratinocytes. Systemic retinoids inhibit the development of new precancerous skin lesions and SCCs in OTRs. The number of new AKs can be reduced by 50 % with the retinoid acitretin in low doses (10-30 mg/day)[159], which is the most widely used regimen. Unfortunately, the benefits are only experienced during treatment. Despite the low dosage, typical adverse events (*e.g.* dry skin, hair loss, pruritus and arthralgias) can lead to discontinuation of the treatment.[160]

- **Systemic therapy for advanced disease**

Skin cancer with advanced disease will only briefly be mentioned as it is not the main objective of this thesis. Until now, no survival benefit has been demonstrated in MM patients with metastatic disease who have been treated with adjuvant chemotherapy, immunotherapy, retinoids or biologic therapy. Recent trials include angiogenesis inhibitors and vaccines.[86] Although systemic chemotherapy is generally not used in the management of SCC, it can potentially be used as an adjuvant or a palliative treatment in locoregionally advanced and metastatic cutaneous SCC.[161]

1.4.4 Radiotherapy

Radiotherapy is the treatment of disease using ionizing radiation. Radiotherapy is effective in the treatment of primary SCC and BCC with response rates similar to those obtained with surgery. However, radiotherapy is generally not considered a first-line therapy due to the high number of treatment sessions needed, healing problems and relatively high recurrence rates.[44, 162] It is mainly indicated in elderly patients with inoperable NMSCs and as an adjuvant therapy following incomplete excision or in tumors with perineural invasion. Postoperative radiotherapy is considered for SCCs that involve regional metastasis.[44] In the management of MM, radiotherapy can be used as adjuvant therapy after lymphadenectomy and as palliative treatment of metastases.[86] Radiotherapy is not recommended in tumors which have previously been irradiated and is also contraindicated in patients with Gorlin's syndrome.[44, 163] Other contraindications include: lesions overlying the lacrimal gland; tumors involving tendon, joint or bone, and lesions located on sites with poor vascularity such as the lower legs.[44]

1.5 Prevention

The constantly rising incidence and the consequent mortality, morbidity and costs of skin cancer call for preventive measures. In medicine, prevention is defined as any activity dedicated to prevent or impede illness and/or reduce the mortality or morbidity from a disease.

1.5.1 Primary prevention

Primary prevention, such as population-based health promotion activities, intends to avoid the development of a disease. Diverse health education campaigns have been carried out in the United States of America, Australia and Europe aimed at reducing sun exposure and sunburn.[75, 164, 165] Sun avoidance, clothing and sunscreens are generally recommended.[44] The routine use of sunscreens, the most commonly used protective measure in all age groups, may reduce the incidence of SCC and has been shown to reduce sunburn and the development of AKs.[166-168]

1.5.2 Secondary prevention

Secondary prevention aims to detect disease early in order to minimize the progression of the disease. Secondary prevention is generally based on patient education to help them recognize skin cancer at an early stage, the recommendation of regular skin self-examination and full body skin examinations by physicians, which may lead to earlier diagnosis and treatment.[80, 81] Despite the fact that such efforts have not yet been proven to reduce the morbidity and mortality of skin cancer[169], most dermatologists consider that this approach must logically be encouraged. Primary and secondary prevention are the focus of Paper IV.

- **The 'Euromelanoma Day' screening campaign**

The 'Euromelanoma Day' screening campaign is an initiative which was launched by a task force linked to the European Academy of Dermatology and Venereology in 2000. The purpose of the 'Euromelanoma Day' is to give the general public a chance to receive skin cancer screening free-of-charge one day a year in early May (Fig. 19). It also involves intense campaigning through television, press, radio and internet in order to educate the public on risk factors, sun protection measures, how to suspect skin cancer and how to perform skin self-examination. Several European countries, including Sweden[170], participate in the campaign every year. Results of the campaign have previously been studied by countries like Belgium, Spain, Greece and Switzerland[165, 171-173], but not by Scandinavian countries, which have high incidence rates of MM and NMSC. The first results of the Swedish national 'Euromelanoma Day' screening campaign are presented in Paper IV.

Figure 19. The slogan for the 2009 'Euromelanoma Day' screening campaign was "There are 101 reasons to have your moles checked" (left). Media was helpful when preparing this public health education campaign in 2008 (below). (Artwork, left: Euromelanoma and Ana Paoli; photo, below: John Paoli)

1.5.3 Tertiary prevention

Tertiary prevention includes the actions taken to reduce the negative impact of a disease that is already present (*e.g.* developing better treatments for MM metastases) or to minimize the risk of developing new primary tumors (*e.g.* chemoprevention). As mentioned above, oral retinoids are used in high-risk patients who have had several previous NMSCs to prevent new precancerous skin lesions and SCCs from appearing. Capecitabine, an orally administered prodrug which is eventually converted to 5-FU especially in tumor cells, is currently being tested to suppress the development of new SCCs in OTRs. Betacarotene supplementation, selenium and low-fat diets have failed to demonstrate a beneficial effect in the chemoprevention of new NMSCs.[67, 167]

2. AIMS OF THE INVESTIGATION

The studies included in this thesis cover selected aspects regarding new techniques for the diagnosis, therapy and prevention of skin cancer with the aim to improve the management of disease. The aims of the investigation were:

- To describe the morphologic and autofluorescent features observed when applying MPLSM for visualization of NMSC *ex vivo*. The lesional features are compared to the morphology seen in images from normal perilesional skin.

- To investigate how men with PIN lesions treated with PDT at Sahlgrenska University Hospital during the period 1999-2004 responded to treatment.

- To evaluate the effectiveness of nerve blocks in relieving pain during PDT for field cancerization in the facial area.

- To describe the demographic characteristics, the risk factors, the clinical diagnoses found and the treatments performed among the patients who were screened for skin cancer during the 'Euromelanoma Day' prevention campaign in Sweden 2008.

3. MATERIALS AND METHODS

3.1 Paper I

Patients

Fourteen patients (8 females and 6 males) with a median age of 65 years (range 47-94 years) with at least one histopathologically verified NMSC were included. This pilot study was carried out prospectively and designed to be descriptive.

Methods

Tissue sample preparation

Standard local excision of 5 SCC *in situ* specimens, 6 superficial BCCs and 3 nodular BCCs was performed. Subsequently, 6 mm punch biopsies were taken from the bulk of the tumor and from normal perilesional skin. The subcutaneous tissue was removed, so that the specimens contained epidermal and dermal tissue. After moistening the tissues with a few drops of phosphate-buffered saline, the samples were placed in an imaging chamber gasket (CoverWell™, Invitrogen™, Eugene, Oregon, USA) adhered to a microscope slide (Menzel-Gläser, Menzel GmbH, Braunschweig, Germany). The slides were wrapped in aluminium foil to protect the samples from photobleaching and stored at 6–8°C until MPLSM was performed.

MPLSM imaging and evaluation

All MLPSM imaging was performed *ex vivo* on fresh tissue samples without adding any fluorophores or staining solutions. Imaging was completed within 4-6 hours after excision and carried out at the Centre for Cellular Imaging at the University of Gothenburg in Gothenburg, Sweden. The MPLSM imaging system comprised a Bio-Rad Radiance 2100MP Rainbow confocal laser *x–y* scanning microscope system (Bio-Rad, Hemel Hempstead, UK; company

purchased by Carl Zeiss in 2004) integrated with an inverted Olympus IX71 microscope (Olympus, Hamburg, Germany). The excitation light, with a wavelength tuned to 780 nm, was produced by a pulsed femtosecond titanium-sapphire laser (Tsunami®, Spectra-Physics, Mountain View, California, USA) with a 10W Millennia Xs pump source. A x 40 water-immersion lens with a 0.8 numerical aperture and a working distance of 1.7mm (Carl Zeiss C Achroplan NIR x 40/0.8) plus an internal descanned photomultiplier tube with a fully opened pinhole were used to detect the emitted autofluorescence in the wavelength region of 450–530 nm. Two-dimensional images with a scan area of 323 x 323 μm were obtained by scanning in a horizontal plane parallel to the skin surface (*x-y* sections) at different depths (*z*-series) with vertical steps ranging from 1-10 μm. Three-dimensional images were created by stacking the *x-y* sections, so-called *z*-stacks. Imaging started at the skin surface continuing down to a maximum depth of 135 μm. Laser power was increased at greater depths to ensure 2PE and autofluorescence emission. The images had a lateral resolution of 0.6 μm and an axial resolution of 3 μm permitting the observation of morphologic structures and fluorescence patterns on a cellular and subcellular level. The resulting digital images were studied using ImageJ 1.34s (National Institutes of Health, USA) and MatLab (MathWorks Inc., Natick, Massachussetts, USA) software.

Follow-up

All patients were followed up after 3-6 months to ensure complete removal of the tumors.

3.2 Paper II

Patients

Eleven males with histopathologically confirmed PIN lesions were treated with PDT during 1999-2004 at Sahlgrenska University Hospital. One patient was excluded as he did not fulfill the inclusion criteria. Thus, 10 patients with a median age of 62 years (range 42-82 years) were included in the study. All lesions were confirmed with histopathological examination. A

thorough history was taken regarding known risk factors for PIN. Three of ten patients had histopathologically confirmed lichen sclerosus, 9 patients were or had been smokers and none of them were circumcised at birth. This study presents a case series in which treatment outcome was studied retrospectively, while HPV detection was performed prospectively after PDT.

Methods

HPV detection and typing

HPV detection and typing was performed at the Department of Medical Microbiology, at Malmö General Hospital in Malmö, Sweden. The technique used was a combination of a general HPV primer (GP5^{+}/6[+]) polymerase chain reaction mediated enzyme immunoassay[174] to detect HPV DNA and a non-radioactive reverse dot blot hybridization[111] for HPV typing. The quality of the extracted DNA for amplification was analyzed using a β-globin PCR.[175] The tissue samples used for HPV detection were punch biopsies from eight patients and swab samples collected from the surface of treated skin areas in two patients who refused further biopsies. In 5 patients, the tissue samples were obtained from existing PIN lesions and, in the other 5 patients, the samples were collected from skin areas without remaining lesions after treatment.

PDT

Nine patients were treated with ALA PDT (described below) and 3 patients with MAL PDT (two patients received treatment with both methods). Patients received a median of 3 PDT sessions (range 1-15). The topical photosensitizing agent (ALA or MAL) was applied under an occlusive dressing (Tegaderm[TM], 3M Corp, Neuss, Germany) during 3 h. The dressing and excess cream was then removed and the treatment area was illuminated with a red light source.

- ALA PDT: ALA cream (20% aminolevulinic acid HCl in Unguentum Merck®) was used as the photosensitizing drug. The light source was a Waldmann PDT 1200L lamp (Herbert Waldmann GmbH, Villingen-Schwenningen, Germany), which provides incoherent light in the range of 600-730 nm, at fluence rates of 35 to 80 mW/cm^2 and with a total fluence of 50 to 80 J/cm^2.

- MAL PDT: The topical photosensitizing agent was 16% MAL cream (Metvix®, PhotoCure ASA, Oslo, Norway), while illumination came from an Aktilite® CL128 light-emitting diode lamp (PhotoCure ASA, Oslo, Norway). The emission spectrum of this red incoherent light was 570-670 nm and the total fluence was 37 J/cm^2 delivered at a fluence rate of 37 mW/cm^2.

Anesthesia prior to illumination

Due to the pain typically caused by PDT, all patients received anesthesia prior to the irradiative phase of PDT. Anesthesia was performed with 5% lidocaine-prilocaine cream (EMLA®, AstraZeneca AB, Mölndal, Sweden) topically, intralesional 1% lidocaine (Xylocain®, AstraZeneca AB) or dorsal penile nerve block with 1% lidocaine (Xylocain®, AstraZeneca AB) injections.

Follow-up

Follow-up was carried out in all patients 9 to 92 months after the last PDT session. Nine of ten patients were followed-up for at least 35 months.

3.3 Paper III

Patients

Sixteen patients (8 men, 8 women) with a median age of 72 years (range 61-86 years) were included in this prospective, open, controlled trial. Patients who had been prescribed PDT for

extensive and symmetrically distributed AKs in the facial area qualified for inclusion. All 16 patients had field cancerization of the forehead. Two patients also had involvement of the upper lip. Another patient presented with AKs on both medial cheeks apart from the AKs on the forehead. One patient was also affected with actinic cheilitis.

Methods

PDT and nerve blocks

This trial involved application of the MAL PDT technique described above in Paper II with unilateral nerve blocks performed on a randomly chosen side of the treatment area 10–15 min before the illumination phase of PDT. Nerve blocks of the supraorbital and supratrochlear nerves were used to anesthetize one side of the forehead; infraorbital blocks numbed the medial cheek and half of the upper lip, while mental blocks were used in the presence of actinic cheilitis. The anesthetic mepivacaine-adrenaline (Carbocain® + adrenaline 10 mg mL^{-1} + 5 μg mL^{-1}, AstraZeneca PLC, London, UK) was used and anesthesia lasted 1-2 hours. Subsequently, the illumination phase of PDT was carried out on both sides of the treatment area simultaneously.

Pain evaluation

A visual analog scale (VAS) was employed to record the maximum pain perceived by the patients during PDT. A VAS score of 0 corresponded to 'no pain' while 10 meant 'worst pain imaginable'. As described by other authors, VAS scores of 0 implied no pain; scores of 1–4 indicated mild pain; 5–6 corresponded to moderate pain, and 7–10 denoted severe pain.[176]

Follow-up

Patients were contacted by telephone within 2 weeks after PDT to obtain information about the pain experienced after PDT and any possible adverse events. Clinical follow-up was carried out after 4–20 weeks to determine the clearance rate of the AKs.

3.4 Paper IV

Patients

One-third of Sweden's dermatologists (n=119) at 34 different clinics and hospitals (10 private clinics and 24 public hospitals) screened 2799 patients. Data from 2659 questionnaires (95 %) were available for analysis. The female:male ratio of the screened patients was approximately 3:2 and the median age of the patients was 57 years (range 5 to 100 years). This study was designed as a descriptive cross-sectional study.

Methods

All Swedish dermatologists (approximately 350) received an invitation to take part in the annual 'Euromelanoma Day' prevention campaign 4 months prior to May 5th, 2008 when the screenings took place. The Swedish Association of Dermatologic Surgery and Oncology prepared a national questionnaire to collect data from the screened population. During the weeks prior to the 'Euromelanoma day', public health education messages were spread through diverse media and people with suspicious lesions were advised to book an appointment for a skin examination at a participating clinic. Patients were charged regular rates for their visits (approximately €30). The first part of the questionnaire contained items regarding patient demographics, the reason for their visit, personal history of skin cancer, familial history of melanoma and skin type. In the second part of the questionnaire, dermatologists reported on the area which was inspected, the presence of any suspicious lesions, the clinical diagnoses found and the treatments provided. In the months after the campaign, participating dermatologists were reminded several times to send anonymous copies of the questionnaires and reports on all histopathologically confirmed MMs to the national coordinator for further analysis.

Follow-up

Patients were not followed up.

3.5 Statistical methods

No statistical analyses were performed in Papers I and II due to the limited amount of data in these studies.

In Paper III, a sample size calculation was performed prior to the study. Earlier studies have shown that pain during PDT follows a normal distribution.[142, 143] The mean VAS scores were expected to be 7.5 with a standard deviation of 2.5 based on our clinical experience. The nerve blocks were assumed to lower VAS scores by 3 points. A power analysis with these estimates resulted in a sample size of 15 patients at a power of 99%. Mean VAS scores from the different treatment sides were compared with paired t-tests (Microsoft Excel, Microsoft Corp., Redmond, Washington, USA). Error limits are presented as standard error of the mean (SEM).

In Paper IV, Fisher's exact test (R 2.8.1, The R Foundation for Statistical Computing, Vienna, Austria) was carried out to analyze categorical data such as the comparison of the number of suspected NMSCs and MMs in patients over and under the age of 50 years.

3.6 Ethics

Papers I-III

The Regional Ethics Committee in Gothenburg, Sweden, approved the studies.

Paper IV

Ethical approval was not considered to be necessary by the Regional Ethics Committee in Gothenburg, Sweden, because all data was anonymous and could not be traced to any individuals.

4. RESULTS

4.1 Paper I

Three-dimensional images of autofluorescent structures were obtained displaying the cell morphology of normal perilesional skin and the 14 NMSCs (Paper I, Figs. 1 and 2).

Normal perilesional skin

Images of all 14 samples of normal perilesional skin exhibited similar morphologic features and were comparable to descriptions by other authors.[106, 177, 178]

SCC *in situ*

Several histopathological criteria of SCC *in situ* were visible in the MPLSM images from the five specimens studied. All lesions presented a thickened *stratum corneum* compared to normal perilesional skin. Hyperkeratosis, manifested by fluorescent nuclei compartments within the corneocytes, was often present. So-called keratin pearls could be seen within the *stratum corneum* as large, rounded bundles of keratin. Within the *stratum spinosum*, multinucleated cells were present and dyskeratotic cells with a higher cytoplasmic fluorescence could be observed. Irregularly distributed keratinocytes and widened intercellular spaces were frequently seen, giving a typical "wind-blown" appearance to the epidermis. Also, pleomorphism and varying nuclei sizes, which are signs of bowenoid dysplasia, were noted. Furthermore, speckled perinuclear fluorescence was visualized to a higher extent within lesions than in the corresponding normal perilesional skin. This feature has previously been described in MPLSM imaging of precancerous epithelial tissues in an *in vivo* hamster model.[179]

Superficial BCC

MPLSM images of the six superficial BCCs also showed typical histopathological features. Hyperkeratosis and a thickened subcorneal epidermis (as mentioned above in the description of the SCC *in situ* lesions) were visible. The tumor cells within the epidermis were monomorphous and typical peripheral palisading of the basal cells could be discerned in a few lesions. The morphologic feature of nuclei polarization with elongated nuclei and cytoplasms in the lower third of the epidermis described earlier in RCLSM images of BCCs was distinguished in one lesion.[98, 180] Moreover, speckled perinuclear fluorescence was observed in the subcorneal epidermis of the superficial BCCs, but also in most of the corresponding normal perilesional skin specimens.

Nodular BCC

The three nodular BCCs imaged by MPLSM *ex vivo* were the most difficult to study due to the thickness of these lesions. The imaging depth was limited to 90-100 µm in two lesions in which only an apparently normal, but slightly thickened, epidermis was visualized. In one lesion, typical nests of basal cells with large oval nuclei, little cytoplasm and classic peripheral palisading were seen in the dermis. Speckled perinuclear fluorescence was not observed in these lesions.

Follow-up

No patients showed clinical signs of recurrence at follow-up 3-6 months after the excision.

4.2 Paper II

Clinical outcome

Complete clinical clearance of PIN lesions was observed a few weeks after PDT in 7 of 10 patients. However, three recurrences occurred after 1, 11 and 14 months, respectively. The four patients with complete clearance without recurrences during a mean follow-up of 35

months underwent 2-8 PDT sessions. Among the three non-responders, two patients only received one PDT session and the third was an especially recalcitrant case which had responded poorly to several therapeutic methods. None of the patients developed invasive SCCs.

Adverse effects

Pain was managed well with the different anesthetic approaches applied. Typical superficial erosions were present after PDT but resolved within a few days.

HPV detection

All tissue samples for HPV detection were obtained after PDT. HPV DNA was only detected in the 5 patients in whom samples were obtained while the PIN lesions were still present. HPV type 18 was found in one patient. In the other four, HPV 16 was detected. One of these patients was also found to have HPV types 45 and 56 in a PIN lesion of the scrotum. Thus, well-known mucosal HPV types were associated with these PIN lesions.

4.3 Paper III

Pain relief

The mean ± SEM VAS score on the non-anesthetized half of all 20 treatment areas was 7.5 ± 0.5 compared to that of the blocked sides which was 1.3 ± 0.3. In other words, pain was reduced by approximately 6 points which was statistically significant ($P<10^{-8}$). When only taking into account treatment of the forehead, the mean VAS scores on the non-anesthetized and blocked side were 7.5 ± 0.6 and 1.2 ± 0.3, respectively, resulting in a clearly significant reduction of around 6 points ($P<10^{-6}$). The nerve blocks also numbed the pain experienced after PDT on the blocked side of the treatment area. This pain reduction lasted approximately 2 hours until the anesthetic effect dissipated.

Adverse events

Nerve blocks were very well tolerated and no adverse effects were reported.

Clinical outcome

Excellent clinical results were noted at follow-up after 4–20 weeks. No differences in treatment outcome were observed when comparing the treatment sides.

4.4 Paper IV

Patients

In total, data from 2659 questionnaires were available for analysis. Almost 60 % of the patients were over the age of 50 and more than 90 % of patients had skin types II-III. Most participants found out about the campaign through the press. The most common reasons for making an appointment were the desire of obtaining a general skin examination and changes in the patient's nevi. Half of the patients had been screened before. Almost 500 patients recalled a personal history of BCC, AKs or Bowen's disease, SCC, atypical nevi, melanoma or other type of skin cancer. Approximately 400 patients reported a familial history of MM.

Clinical examination

Dermatologists performed full body skin examinations in more than 60 % of the patients. The most common diagnoses found were benign nevi (37.7 %) and SKs (36.7 %). More than 800 patients presented with atypical nevi or cancerous lesions. NMSCs (including AKs) were identified in approximately 450 patients, whereas MM was only clinically suspected in 18 patients. In patients above the age of 50 years, skin cancer was suspected in 27.2 % of all patients but only in 3.3 % of those under this age ($P<10^{-5}$).

MM detection

Twenty-four histopathologically confirmed invasive MMs (n=14) and MM *in situ* lesions (n=10) were detected among the screened patients, resulting in a detection rate of 0.9 %. Eighteen of these lesions were diagnosed in women. The majority of the 14 invasive MMs presented histopathological characteristics associated with a relatively good prognosis (8 were thinner than 1 mm and 11 had a Clark level of II or III). Only one of these MMs presented with the negative prognostic feature of ulceration.

Treatments performed

In total, over 1200 patients needed therapy, follow-up or referral to another physician and around 350 of these patients required multiple treatments. Although most treatments were carried out on the screening day, a new visit was necessary to provide appropriate medical care for approximately 390 patients. Cryosurgery was the most common procedure performed (329 patients). In total, 508 lesions were biopsied or excised.

5. DISCUSSION

5.1 Paper I

Methodological considerations

To our knowledge, this was the first study on MPLSM imaging of NMSCs. The study was therefore designed as a descriptive study, which is why no sample size calculations were performed.

MPLSM imaging was performed *ex vivo* since our MPLSM system is not approved for *in vivo* imaging. Theoretically, *ex vivo* imaging is less efficient since the autofluorescence of the endogenous fluorophores in the epidermis and upper dermis decreases as the metabolic activity has disappeared. In order to avoid photobleaching and to mimic *in vivo* conditions, samples were protected with aluminium foil and all imaging was carried out within 4-6 hours after the specimens were obtained from surgery.

The lateral and axial resolution of 0.6 µm and 3 µm, respectively, allowed for the visualization of cellular and subcellular structures. This resolution is comparable to that of RCLSM (lateral resolution = 0.5-1.0 µm, axial resolution = 3–5 µm). However, it is considerably higher than the resolution of OCT (axial resolution = 8 µm, lateral resolution = 24 µm). The limited imaging depth of approximately 100 µm unfortunately made the study of thicker nodular BCCs more difficult. Other MPLSM systems can acquire images at depths of up to 200 µm.[109] In comparison, RCLSM can achieve images down to 250 µm.[99] Meanwhile, OCT has a maximal penetration depth in skin of 2.0-2.5 mm.[97]

General discussion

Three-dimensional MPLSM images of NMSCs revealed traditional histopathological diagnostic criteria. Imaging with this technique did not require any exogenous chemicals such as histopathological stains. The morphologic features observed in SCC *in situ* lesions, superficial BCCs and in one of three nodular BCCs differed substantially from the corresponding perilesional skin. MPLSM is an imaging technique which potentially could provide bedside, non-invasive, histopathological diagnosis of skin cancer and other skin diseases in the future. However, several safety issues and limitations must be surpassed for this to be possible (see section 7).

5.2 Paper II

Methodological considerations

Very few patients with PIN were treated with PDT at Sahlgrenska University Hospital during the period 1999-2004. Thus, the number of patients included in the study was limited. At that time, there were no clear protocols for PDT in the treatment of PIN, which explains the disparate number of PDT sessions applied. Treatment outcome may have been affected by the varying degrees of cellular atypia presented; by the two different combinations of photosensitizing agent plus light source which were used and/or by the dissimilar number of PDT sessions given. As for HPV detection, the results may have been influenced by the fact that tissue samples were collected from PIN lesions in some cases and from healthy skin in others. Moreover, tissue sampling was performed with two different methods (*i.e.* biopsy or swab sampling).

General discussion

PIN in males over 40 years of age should be considered premalignant lesions with a risk of progression to invasive SCC ranging 5-33 %.[32, 181] There are no ideal therapeutic alternatives for PIN, but non-mutilating methods are preferred.[33] The multifocal nature of HPV infection

and PIN lesions may explain the recurrences after surgical and destructive methods which are only applied in the visibly affected areas. Therefore, field therapies such as PDT are an interesting approach to this condition. Prior to our study, only a handful of case reports of PDT for PIN had been published[182-186] making our study the largest case series to date at the time of its publication. Since then, another case series of 10 patients was published in 2007 with similar results to those presented in our study.[187] PDT has also been investigated for the treatment of vulvar intraepithelial neoplasia with promising results.[188, 189] These reports do not allow us to draw any firm conclusions about the effectiveness of PDT for the treatment of PIN, but they may be helpful in the design of future prospective trials so that an adequate protocol can be determined.

Other well-known risk factors (*i.e.* lichen sclerosus, smoking and lack of neonatal circumcision) were present in the patients. Physicians should therefore provide long-term follow-up of lichen sclerosus and encourage patients to quit smoking. Neonatal circumcision remains controversial.

Pain management during PDT is essential (see further in Paper III). Although PIN lesions present a challenge in this sense, dorsal penile nerve blocks with complementary local infiltration anesthesia if necessary effectively relieved pain in our patients. Erosions, swelling and erythema caused by PDT healed within 5-10 days and the cosmetic results in patients with complete clearance was excellent. Furthermore, no invasive SCCs developed in the patients in our study. Long-term clinical follow-up and routine self-inspection of all patients with PIN is recommended regardless of which treatment has been given due to the risk of recurrence and progression to invasive SCC.[33] Urologists should be consulted when PIN lesions grow near or inside the urethra.

5.3 Paper III

Methodological considerations

This study was designed as an open trial due to the fact that it is difficult to blind the patient or the physician when performing a nerve block. The "split-face" nature of the study allowed each patient to serve as his/her own control. This was advantageous as the subjective experience of pain is variable for different individuals. Patients had no problem discerning the pain perceived on the two treatment sides regardless of the treatment area's location. The side to which the nerve block was applied was randomly chosen as the exact amount of actinic damage could theoretically have been slightly different between the two sides of the treatment area. The experience of pain is purely subjective and cannot be measured objectively; however, the use of a VAS is one of the most commonly used measures of pain intensity in clinical trials. The method's validity for the assessment of pain intensity is supported by a great deal of evidence.[190]

General discussion

Nerve blocks significantly reduce pain when treating extensive facial AKs with PDT, especially when treating field cancerization of the forehead. The mean VAS scores on the non-anesthetized side could be translated into severe pain, whereas the low VAS scores on the blocked side reflected mild pain at most. The mild pain perceived on the blocked side was most often due to the presence of AKs outside of the anesthetized field according to the patients. In theory, it could also be explained by incomplete anesthesia of the treatment area. VAS scores were reduced by more than 6 points in general and all patients with moderate to severe pain reduced their VAS scores by at least 50 %. Patients also benefitted from pain reduction after PDT.

There is further support for the use of nerve blocks when managing pain during PDT. As mentioned earlier, dorsal penile nerve blocks effectively relieved pain in patients treated with

PDT for PIN lesions. Furthermore, we have recently published a study in which excellent pain relief was achieved with forehead and occipital nerve blocks when treating field cancerization of the forehead and scalp with PDT.[191] In that study, the local anesthetic bupivicaine-adrenaline (Marcain®adrenalin 5mg ml^{-1} + 5µg ml^{-1}, AstraZeneca AB, Södertälje, Sweden) was used. This provided anesthesia during 2-6 hours which further prolonged the pain relief after PDT. Nerve blocks are simple procedures to perform with a very low risk for complications.[157, 158, 192] In fact, no adverse effects were noted in our patients. It is also noteworthy that nerve blocks did not interfere with the effectiveness of PDT. After completion of this trial, nerve blocks were incorporated into routine practice at Sahlgrenska University Hospital. Over 300 patients have now been treated with the same effective pain relief and safety as observed in this study.

5.4 Paper IV

Methodological considerations

Data regarding patient demographics and risk factors, the diagnoses detected and the treatments performed were retrieved thanks to the introduction of a national questionnaire. In order to recruit a higher number of participating dermatologists, the number of items on the questionnaire was limited. Thus, questions regarding risk factors such as previous UV light exposure and the presence of a high number of melanocytic nevi were excluded. Full body skin examinations are generally recommended when screening for skin cancer[193] but should also be complemented with dermoscopy.[194] The questionnaire used in this study did not contemplate the use of dermoscopy but the majority of patients were screened by full body skin examinations. A strength of this study was that all reports on detected MMs were based on histopathological confirmation. More MMs were possibly found but not reported or lost due to referrals to other physicians, but this theoretical loss of data should be limited as most excisions were performed on the screening day. Apart from the MMs, all data on diagnoses were based solely on clinical suspicion. Two-thirds of the treatments performed on the

screened patients were carried out on the screening day, which minimized the risk of long waiting lists after the campaign. Data on the medical procedures received at a later date were unfortunately not available.

General discussion

The rising incidence of MM and NMSC in Sweden must be reversed. Primary and secondary prevention of MM and NMSC through changes in the population's behaviour in the sun and increased early detection of the tumors are theoretically the most efficient way of reducing morbidity and mortality.[44, 195] The 'Euromelanoma Day' campaign is mainly based on early detection through screening. However, this campaign is also dedicated to educating the general public about the importance of sun protection measures, skin self-examination and other warning signs of skin cancer (*e.g.* the "ABCDE" criteria, the "ugly duckling sign" or "non-healing ulcers"). The United States Preventive Task Force states that there is insufficient evidence that skin cancer screening can reduce mortality and morbidity.[169] However, this can be explained by the fact that extremely large and expensive randomized trials including hundreds of thousands of subjects are required to provide such evidence.[196]

As observed in similar campaigns, women were overrepresented among the screened population.[165, 197] Campaign advertising should be directed towards men in a greater degree, as they have the same risk as women for MM and even higher incidence rates of NMSC. In order to increase the effectiveness of skin cancer screening campaigns, it is important to attract high-risk patients.[198] This goal was seemingly accomplished since more than 50% of the participants complained of changes in their moles or non-healing ulcers. The screened patients were also approximately 10 years older in the Swedish campaign compared to campaigns in Greece and Switzerland.[171, 173] A substantial number of NMSCs were detected, which is probably explained by the older age of the Swedish participants. Over 6 % of the patients had BCCs compared to detection rates of 2.1-4.2 % in earlier campaigns in Belgium,

Greece and Switzerland.[165, 171, 173] Individuals without suspicious lesions may have been reluctant to make an appointment since the examination was not free of charge.

In regards to the treatment, the high number of AKs and seborrhoeic keratoses diagnosed probably explain why cryosurgery was the most common medical procedure. Over 500 biopsies or excisions were performed, but it was not possible to collect data from all corresponding histopathological evaluations. In total, 120 melanocytic lesions were excised. During screening campaigns, there is a potential risk of unnecessary treatment of benign lesions with a subsequent increase in costs and harm to the patients.[193, 198] On the other hand, dermatologists at specialised skin cancer clinics need to excise 10 to 29 melanocytic lesions to detect one melanoma in routine practice.[199-201] Considering the fact that 24 MMs were confirmed, the number of excisions carried out on these patients does not seem excessive.

Twenty-four histopathologically confirmed MMs were reported despite only 18 questionnaires declaring this clinical suspicion. This is probably due to an 'innocent until proven guilty' policy among participating dermatologists. The median age of MM patients matched the median age of other MM patients in Sweden (63 and 58 years for men and women, respectively). Detection rates for MM in 'Euromelanoma Day' campaigns in Greece, Spain and Switzerland were 0.25-0.35 % compared to the relatively high detection rate of 0.9 % observed in the Swedish and the Belgian campaigns.[165, 171-173] Three-fourths of the MMs were thin (<1 mm) or *in situ* implying a relatively early detection in most cases.

6. CONCLUSIONS

- The morphologic and autofluorescent features of SCC *in situ* and superficial BCCs imaged with MPLSM *ex vivo* differ from normal perilesional skin and reveal typical histopathological criteria for these diagnoses. Nodular BCCs are difficult to study with this method due to the limited imaging depth.

- PDT is a therapeutic option which may be considered in the management of PIN lesions if first-hand alternatives such as excision or laser ablation are not warranted. However, larger prospective studies are needed to establish a proper treatment protocol.

- Nerve blocks provided excellent pain relief during PDT for field cancerization of the forehead and other facial areas without affecting treatment effectiveness.

- The 'Euromelanoma Day' screening campaign in Sweden 2008 attracted more high-risk patients than similar campaigns in other countries. It rendered high pick-up rates of NMSC and MM, while the MMs detected were predominantly of favorable prognosis.

7. OUTLOOK FOR THE FUTURE

This thesis covers four measures which can be used to improve the diagnosis, treatment and prevention of skin cancer. This field of research is seemingly limitless providing a wide range of possibilities for further improvements. However, in regards to the aspects described in this thesis, the following research prospects are of interest.

MPLSM

We have shown that specific histopathological criteria for superficial NMSCs can be observed with MPLSM *ex vivo*. Consequently, a larger prospective study should be designed to determine the specificity and sensitivity of the morphologic features found with MPLSM in superficial BCCs and SCC *in situ*. Our study investigated autofluorescence of endogenous fluorophores, but the possibility exists of combining this with PpIX fluorescence after application of ALA or MAL to the lesions. The MPLSM images would hopefully yield a clearer visualization of cancerous cells as these produce PpIX to a higher extent. Another approach would be to combine MPLSM with other non-invasive imaging techniques (*e.g.* OCT and RCLSM) to increase the diagnostic accuracy.

MPLSM could become an *in vivo*, bedside, non-invasive, optical skin biopsy technique, but several aspects of the technique must be improved. One of the potential risks of MPLSM is tissue damage. Fortunately, most endogenous fluorophores in human skin have low one-photon absorbance in the NIR range. Melanin, however, can cause thermal tissue damage at the dermo-epidermal junction due to one-photon effects.[202] Eventual tissue damage should otherwise originate from non-linear effects (*e.g.* 2PE). Moreover, femtosecond-laser irradiation can produce photochemical effects similar to the DNA damage obtained after UV exposure.[203] Thus, safe laser power ranges for MPLSM must be properly investigated in order to avoid undesirable tissue damage.

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As mentioned earlier, the imaging depth with MPLSM is currently insufficient to study thick, invasively growing tumors. Laser power would have to be greatly increased to image deeper structures which could lead to tissue damage. When imaging NMSCs, the imaging of dermal structures is also limited by the presence of hyperkeratosis, parakeratosis and a thickened acanthotic epidermis. These elements could possibly be minimized with so-called optical clearing agents (*e.g.* glycerol, propylene glycol and glucose), which reduce the scattering properties in tissue.[204] The use of optical clearing agents may facilitate the imaging of deeper structures, but it is unknown if these agents could affect tissue morphology.

Currently, MPLSM is also time-consuming as scans take approximately 10-15 minutes.[109] Furthermore, sampling of several areas within a lesion may be necessary. In a clinical setting, image acquisition must be quickened in order to avoid distortion due to movements made by the patient. The method must also be made faster to gain acceptance among physicians. High-speed MPLSM has been tested for cytometry of skin tissue but was difficult to perform due to the high turbidity of human skin and the low autofluorescence signal at high speed.[205] Furthermore, the process of interpretation of MPLSM data may be more efficient if image analysis is performed.[206]

Surface irregularities and difficult-to-reach skin areas are also a challenge for current MPLSM systems such as the only commercially available system *DermaInspect®*.[109] Objectives as small as 2 mm in diameter are being developed[207] and may allow for MPLSM imaging of such areas. The axial resolution in MPLSM should also be improved, which requires corrections for the varying optical properties of human skin.[208] Finally, MPLSM systems are very expensive mainly due to the advanced laser equipment which is needed. The development of low-cost lasers for MPLSM is currently progressing[209], which may reduce costs significantly in the future.

PIN and PDT

Currently, surgery and CO_2-laser ablation are considered first-line therapeutic modalities in the treatment of PIN.[33] Our cases series demonstrated that PDT is an alternative option for the management of PIN lesions in middle-aged and elderly men. Another recent case series including 10 patients presented similarly promising results using MAL PDT.[187] Interestingly, 8 patients only received one treatment in this study compared to a median number of PDT sessions of 3 in ours. Hence, further studies are needed to establish a standard protocol for this treatment on PIN lesions. Furthermore, randomized controlled studies should be carried out to compare the effectiveness of PDT with first-line therapies. Another way to confront the multifocal nature of PIN lesions may be to combine therapies. Studies have shown excellent results with combinations of 5-FU, CO_2-laser ablation plus interpheron alpha-2a (an antiproliferative and antiviral drug).[210] It would be interesting to compare the effectiveness and recurrence rates achieved with a lesion-specific treatment modality (*e.g.* surgery or CO_2-laser ablation) with that of a lesion-specific treatment modality followed by PDT to target subclinical disease.

Nerve blocks and PDT

After many years of suffering, patients receiving PDT for field cancerization in the facial area can now be offered excellent pain relief with supraorbital/supratrochlear, infraorbital and mental nerve blocks. As mentioned earlier, our group has also published similar results attained when treating field cancerization of the forehead and scalp by combining supraorbital/supratrochlear and occipital nerve blocks.[191] The next logical step would be to find new areas of the skin in which peripheral nerve blocks could be used to relieve pain during PDT. One such area may be the dorsum of the hands, which are commonly affected with field cancerization in chronically sun-damaged patients and OTRs. The use of regional nerve blocks of the radial, ulnar and median nerves at the wrist may be useful in this area. Unfortunately, nerve blocks cannot easily be applied on all skin areas which can be affected with field cancerization (*e.g.* the chest, the lower arms or the lower legs). Other pain

management strategies should be researched for these body sites. For instance, reduced VAS scores seem to be obtained when PDT is performed using natural sunlight as an alternative light source.[211] Furthermore, nerve blocks could be applied during PDT for other indications than NMSC. One such example is PDT for facial acne vulgaris in which moderate to severe pain is the norm.[212-214]

Skin cancer prevention

The Euromelanoma Task Force within the European Academy of Dermatology and Venereology is currently increasing the collaboration between the countries participating in the 'Euromelanoma Day' screening campaign. Ten countries participated in the first campaign in 2000, whereas 26 countries will be participating in the 2009 edition. In this year's campaign, a unique questionnaire and common follow-up instructions are being incorporated, which will provide comparable data from the participating countries. If an average of 3000 patients are screened per country, data from 78 000 patients could potentially be available for further analysis.

The questionnaire includes items concerning the most important risk factors and also covers some risk factors which were excluded from the Swedish campaign in 2008 (*e.g.* previous UV light exposure and the presence of a high number of melanocytic nevi). International differences in the diagnostic procedures employed may also be reflected in this study. We may be able to determine how widespread the use of full body skin examinations or dermoscopy is during screening in different European countries. For the first time, an attempt will be made to collect the histopathological reports of all suspicious lesions which were biopsied or excised as a consequence of the screenings. Thus, more reliable data on the detection rates for MM and NMSC will be obtained. Additionally, national campaigns often present slight organizational variations (*e.g.* free screening *vs* charging regular rates, age limits or advertising targeted at certain individuals), which could affect the risk profile of the

screened patients. Thus, comparable data between the countries may provide helpful information for future campaign planning.

On another note, the introduction of HPV vaccines against high-risk mucosal HPV types 16 and 18 will hopefully minimize the risk of cervical cancer.[69] This could potentially also lower the incidence of PIN. As mentioned before, these vaccines are still not approved for male individuals, but initial trials have shown positive results in the prevention of external genital lesions in young men.[215] Furthermore, studies regarding the etiological role of betapapillomaviruses (*e.g.* HPV types 5 and 8) in the development of SCC, especially in OTRs, are needed. Vaccines against these HPV types could also theoretically decrease the incidence of cutaneous SCCs.

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