Epidemiology, diagnostics and treatment of non-melanoma skin cancers

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Do not go gentle into that good night,
Old age should burn and rave at close of day;
Rage, rage against the dying of the light.

Though wise men at their end know dark is right,
Because their words had forked no lightning they
Do not go gentle into that good night.

Good men, the last wave by, crying how bright
Their frail deeds might have danced in a green bay,
Rage, rage against the dying of the light.

Wild men who caught and sang the sun in flight,
And learn, too late, they grieved it on its way,
Do not go gentle into that good night.

Grave men, near death, who see with blinding sight
Blind eyes could blaze like meteors and be gay,
Rage, rage against the dying of the light.

And you, my father, there on the sad height,
Curse, bless, me now with your fierce tears, I pray.
Do not go gentle into that good night.
Rage, rage against the dying of the light.

– Dylan Thomas, 1951

Abstract

Skin cancer, including malignant melanoma and non-melanoma skin cancer (NMSC), is a growing problem due to the increasing incidence in Sweden and in other Caucasian populations. NMSCs are diagnosed as often as all other cancers combined and include basal cell carcinoma (BCC), squamous cell carcinoma (SCC), precursors to SCC such as Bowen's disease (BD) and actinic keratosis (AK), as well as several rare skin cancers including Merkel cell carcinoma (MCC). The purpose of this thesis was to investigate novel aspects within the fields of epidemiology, diagnosis and treatment of NMSCs.

In study I, the incidence and clinical characteristics of Swedish patients with MCC was explored. During the study period from 1993 to 2012, the age standardised incidence of MCC almost doubled with an increase of 73-85 % depending on the population used for age standardisation. The overall incidence for women and men per 100,000 persons, using the world population for age standardisation, rose from 0.11 to 0.19 between 1993 and 2012.

In study II, the effectiveness of photodynamic therapy (PDT) for the treatment of BD was evaluated retrospectively for 423 lesions in 335 patients. The study showed that PDT was a relatively effective treatment with a complete clearance rate of 63.4 % after a median follow-up time of 11.2 months. BD lesions greater than 20 mm in size and a single session of PDT were factors associated with statistically worse outcome.

In study III, a novel illumination protocol in PDT for multiple AKs using a stepwise increase of light intensity, staying below 50 mW/cm^2 during the whole treatment session, was compared to the conventional illumination protocol to assess pain levels during treatment and effectiveness. Both protocols had the same total light dose of 37 J/cm^2. The novel treatment protocol led to a small but statically significant decrease in pain (Δ 1.1 points on a visual analogue scale, $p<0.01$). However, the clearance rate with the new protocol was slightly but significantly lower than that of the conventional protocol (91.2 % vs 93.7 %, respectively) ($p=0.04$).

In study IV, the chemical composition of lipids in BCCs was mapped using Time-of-Flight-Secondary-Ion-Mass-Spectrometry (ToF-SIMS). ToF-SIMS was able to identify different lipids in healthy and cancerous tissue. Furthermore, sphingomyelin lipids were found in aggressive BCCs whereas phosphatidylcholine lipids were observed in less aggressive tumours.

In conclusion, the incidence of MCC has increased the last 20 years, PDT is a relatively effective treatment modality in BD, novel illumination protocols with lower light intensity can decrease pain in PDT and ToF-SIMS can be used to identify the lipid composition of BCCs.

Keywords: non-melanoma skin cancer, basal cell carcinoma, Merkel cell carcinoma, Bowen's disease, imaging mass spectrometry, lipidomics, photodynamic therapy, pain.

Sammanfattning på svenska

Hudcancer, innefattande malignt melanom och icke-melanom hudcancer (förkortat NMSC på engelska från begreppet ”non-melanoma skin cancer”), är ett växande problem på grund av den ökande incidensen i Sverige och i andra kaukasiska populationer. NMSC diagnostiseras lika ofta som alla andra cancerformer kombinerat och innefattar basalcellscancer (BCC), skivepitelcancer (SCC) samt flera sällsynta former av hudcancer såsom Merkelcellskarcinom (MCC). Andra mycket vanliga former av NMSC är de olika förstadierna till SCC: Bowens sjukdom (förkortat BD från engelska namnet ”Bowen’s disease”) och de allra tidigaste förstadierna som kallas aktiniska keratoser (AK). Syftet med denna avhandling var att undersöka nya rön inom epidemiologi, diagnostik och behandling av NMSC.

I studie I undersöktes förekomst och kliniska karaktäristika hos svenska patienter med MCC. Under studietiden från 1993 till 2012 fördubblades nästan den åldersstandardiserade incidensen av MCC med en ökning på 73 - 85% beroende på vilken population som användes för åldersstandardisering. Den totala incidensökningen för både män och kvinnor, baserad på världspopulationen för ålderstandardisering, steg från 0.11 till 0.19 fall per 100 000 invånare under tiden som studien pågick. Majoriteten av alla MCC hittades på solexponerade kroppsdelar, dvs ansikte och hals, vilket stöder teorin om att ultraviolett strålning är en viktig bidragande riskfaktor för utvecklandet av MCC.

I studie II utvärderades effektiviteten av fotodynamisk terapi (PDT) vid behandling av BD. PDT innebär kortfattat att man stryker på en kräm på området som ska behandlas och därefter belyser man med rött ljus. Krämen innehåller aminolevulinsyra eller dess metylester (metylaminolevulinat) som sedan omvandlas inom hudcellerna till ett ljuskänsligt porfyrinämne. Efter att krämen varit på huden i tre timmar belyser man med synligt rött ljus. Energin från ljuset reagerar med porfyrinet och behandlingen börjar verka. De sjuka cellerna (tumörcellerna) förstörs. Studie II visade att PDT var en relativt effektiv behandling med en komplett utläkning på 63,4% efter en uppföljningstid på 11,2 månader. Två riskfaktorer visade sig vara förknippade med statistiskt sämre utfall: lesioner större än 20 mm i diameter och en enda session av PDT istället för de numera sedvanliga två sessionerna.

I studie III bedömdes ett nytt belysningsprotokoll för PDT vid aktinisk keratos med avseende på effektivitet och upplevd smärta. En stegvis ökning av ljusintensiteten i detta nya belysningsprotokoll jämfördes med det konventionella bestrålningsprotokollet. Båda protokollen hade samma totala ljusdos på 37

J/cm^2. Det nya behandlingsprotokollet ledde till en liten men statiskt signifikant minskning av upplevd smärta med 1,1 poäng på en visuell analog skala (ett smärtskattningsinstrument där ’0’ är lika med ingen smärta alls och ’10’ motsvarar värsta tänkbara smärta). Det nya protokollet var dock aningen mindre effektivt med en statistiskt signifikant lägre utläkningsfrekvens än det konventionella protokollet (91,2% respektive 93,7% utläkta AK).

I studie IV kartlades den kemiska sammansättningen av lipider (fetter och fettliknande ämnen) i BCC med användning av en avancerad teknik som kallas ”Time-of-Flight-Secondary-Ion-Mass-Spectrometry” (ToF-SIMS). ToF-SIMS kunde reproducerbart identifiera olika lipider i normal hud respektive i hud med BCC. Sfingolipider observerades i högaggressiva BCC medan fosfatidylkolinlipider sågs i lågaggressiva BCC.

Sammanfattningsvis visar avhandlingen att:

- Incidensen av MCC har ökat de senaste två decennierna i Sverige.
- PDT är en relativt effektiv behandlingsmetod för BD.
- Smärtan vid PDT kan minskas med nya belysningsprotokoll med lägre ljusintensitet vid behandling av AK.
- ToF-SIMS kan kartlägga den kemiska sammansättningen av lipider i BCC.

Medvetenheten om ökningen av MCC har lett till att både vårdpersonal och patienter oftare misstänker MCC vilket är av stor betydelse för tidigare diagnos och bättre överlevnad. PDT fortsätter att ha en plats i behandlingsarsenalen vid BD och AK på hudkliniker i Sverige och övriga världen. Resultaten i denna avhandling visar dock att mer noggrann uppföljning kan behövas efter behandling av BD med PDT samt att större lesioner kan lämpa sig bättre för andra behandlingar. Smärtan vid PDT går att minska med nya belysningsprotokoll men protokollet som presenteras i denna avhandling gör inte detta i tillräckligt stor grad för att vara kliniskt relevant. Kartläggning av lipidsammansättning med ToF-SIMS i både hög- och lågaggressiva BCC kan vara av stor vikt för framtida läkemedelsutveckling och utveckling av nya diagnostiska metoder.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Zaar O, Gillstedt M, Lindelöf B, Wennberg-Larkö AM, Paoli J.
Merkel cell carcinoma incidence is increasing in Sweden
J Eur Acad Dermatol Venereol. 2016 Oct;30(10):1708-1713.

II. Zaar O, Fougelberg J, Hermansson A, Gillstedt M, Wennberg A-M, Paoli J.
Effectiveness of photodynamic therapy in Bowen's disease: a retrospective observational study in 423 lesions
J Eur Acad Dermatol Venereol 2017 Aug;31(8):1289-1294.

III. O. Zaar, A. Sjöholm Hylén, M. Gillstedt, J. Paoli
A prospective, randomised, within-subject study of ALA-PDT for actinic keratoses using different irradiation regimes
Submitted for peer review.

IV. Munem M, Zaar O, Dimovska Nilsson K, Neittaanmäki N, Paoli J, Fletcher JS.
Chemical imaging of aggressive basal cell carcinoma using time-of-flight secondary ion mass spectrometry
Biointerphases. 2018 Jan 12;13(3):03B402. E-publication ahead of print.

Content

Abbreviations

AK ..... Actinic keratosis
ALA ..... 5-aminolevulinic acid
BCC ..... Basal cell carcinoma
BD ..... Bowen's disease
C&E ..... Curettage and electrodesiccation
DESI ..... Desorption electrospray ionization
D-OCT ..... Dynamic OCT
EMA ..... European Medicines Agency
FDA ..... Food and Drug Administration
FU ..... Follow-up
GCIBs ..... Gas cluster ion beams
HD-OCT ..... High-definition OCT
Hh ..... Hedgehog
HRUS ..... High resolution ultrasound
H&E ..... Hematoxylin and eosin
ITO ..... Indium tin oxide
laBCC ..... Locally advanced basal cell carcinoma
LEDs ..... Light-emitting diodes
MAL ..... Methyl aminolevulinate
MALDI ..... Matrix-assisted lased desorption ionization mass spectrometry
MAF ..... Maximum Autocorrelation Factor
mBCC ..... Metastatic basal cell carcinoma
MCC ..... Merkel cell carcinoma
MCPyV ..... Merkel cell polyomavirus
MM ..... Malignant melanoma
MMS ..... Mohs micrographic surgery
MPLSM ..... Multiphoton laser scanning microscopy
NMSC ..... Non-melanoma skin cancer
OCT ..... Optical coherence tomography
PC ..... Phosphatidylcholine
PDT ..... Photodynamic therapy
PpIX ..... Protoporphyrin IX
PTCH1 ..... Patched 1
PUVA ..... Psoralen and UV-A
RCM ..... Reflectance confocal microscopy
ROS ..... Reactive oxygen species
RS ..... Raman spectroscopy
SCC ..... Squamous cell carcinoma
SCR ..... Swedish Cancer Registry
SM ..... Sphingomyelin
SMO ..... Transmembrane protein Smoothened
ToF-SIMS ..... Time-of-Flight Secondary Ion Mass Spectrometry
UVR ..... Ultraviolet radiation
VAS ..... Visual analogue scale
5-FU 5-Fluorouracil

1. Introduction

1.1 The human skin

Human skin is one of the largest organs in the body with an area of 1.5-2.0 m^2 and also one of the heaviest with a weight of 4-5 kg [1]. The skin is a semipermeable barrier that is responsible for many life-sustaining functions such as keeping the internal human environment stable and all the other organs of the body in place. Other functions of the skin include photoprotection, sensory input, thermoregulation as well as immunological recognition and activation among others [1, 2].

The skin is divided into three different layers: epidermis, dermis and subcutis *(Fig. 1)*. The thickness of the most superficial layer, the epidermis, can vary from approximately 0.05 mm on the eyelids up to 1.0 mm on the soles of the feet. It consists of mainly three different cell types: keratinocytes, melanocytes and Langerhans' cells. The majority of the cells are keratinocytes, which undergo a maturation process starting at their origin in the so-called basal layer at the epidermal border towards the dermis. The keratinocytes mature as they progress towards the surface of the skin while shedding their cell nucleus while simultaneously forming an acellular barrier at the surface of the skin (the stratum corneum). The epidermal turnover process takes approximately 52-75 days in normal skin but can be altered during damage to the skin or when inflammation is present. In psoriasis, for example, total epidermal renewal is only 8-10 days [3]. Melanocytes protect the skin by forming the pigment melanin in organelles called melanosomes, which in turn transfer melanin to nearby keratinocytes via the melanocyte's dendrites, ultimately absorbing ultraviolet radiation (UVR). Lastly, the Langerhans' cell is also a dendritic cell which functions as an antigen-presenting cell and, when activated, travels from the epidermis to local lymph nodes where it can activate T-cells and start an immune response [1, 2].

A more uncommon cell in the epidermis is the Merkel cell, which serves as a sensory receptor relaying information regarding texture and pressure to the brain. Merkel cells are believed to originate from the epidermal lineage and are located in the basal layer of the epidermis [4]. In addition, they produce certain hormones and are thus referred to as neuroendocrine cells [1, 2].

The next layer of skin, the dermis, is vascularized in contrast to the epidermis. Structure and nutrients are provided by the dermis. It consists of a gel of polysaccharides and a matrix with elastin and collagen fibers. The most important cells of the dermis are dermal dendritic cells, mast cells and macrophages, which all play different parts in immunity and inflammation along with fibroblasts, which produce the components of the polysaccharide gel and fibrous

matrix. Nerves, hair follicles as well as different blood and lymphatic vessels can also be found in the dermis. The thickness of the dermis is roughly tenfold that of the epidermis ranging from 1 to 10 mm depending on the body part [1, 2].

The subcutis, or subcutaneous tissue, consists mainly of adipocytes and functions as thermal insulation, protection from external trauma and as an energy reserve [1, 2]. There are also vessels and nerves residing and passing through this layer of the skin. The lipids of the skin are first and foremost made up of cholesterol, free fatty acids, ceramides and, to a lesser extent, of cholesterol esters, diacylglycerol and triacylglycerol [5, 6].

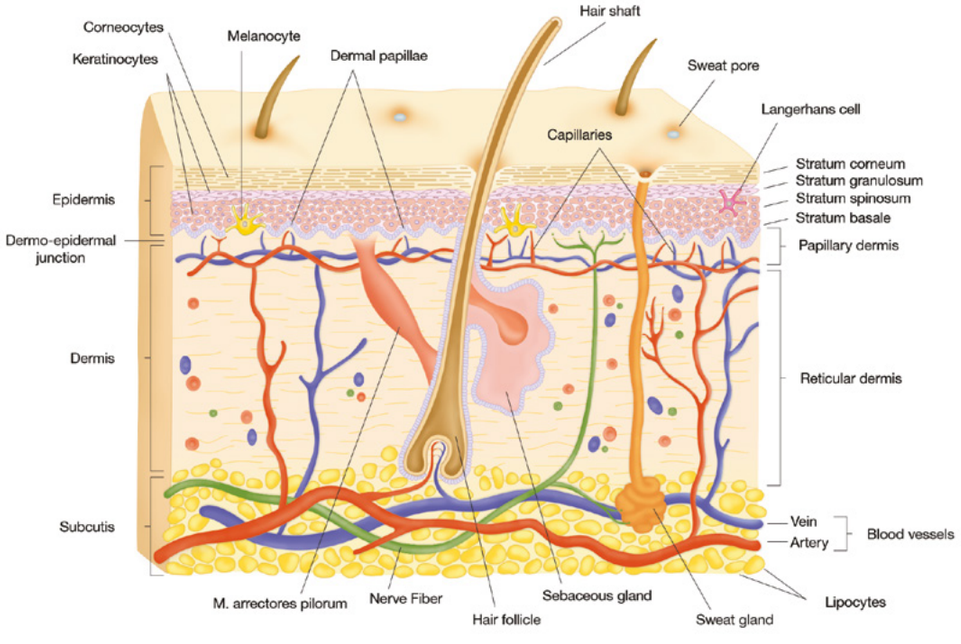


FIGURE 1. The anatomy of the skin. Artwork by Ana Paoli, reprinted from John Paoli's thesis "Selected aspects on improving the management of skin cancer" (University of Gothenburg, 2009) with permission from the author.

1.2 Skin cancer

Skin cancer, including malignant melanoma (MM) and non-melanoma skin cancer (NMSC), is a growing problem with increasing incidence in Sweden and in other Caucasian populations [7, 8]. The incidence rate of MM in Sweden has been rising by just over 5 % per year during the 21st century with an incidence in 2015 of 41.6-36.3 (male-female) per 100,000 persons adjusted for the age of the Swedish population in 2000 [9]. The same year, 3,951 new cases of MM were registered in the Swedish Cancer Registry (SCR), which corresponds to 6.0 % of all diagnosed cancers [9]. Although MM is a fascinating type of skin cancer, this diagnosis will not be discussed in detail here since it is beyond this thesis' scope.

1.2.1 Non-melanoma skin cancer

NMSCs are diagnosed as often as all other cancers combined and include squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and several rare skin cancers such as Merkel cell carcinoma (MCC) [9]. The origin of these cancers ranges from keratinocytes in different stages of maturation to rarer cell types such as Merkel cells. Nowadays, NMSCs are commonly referred to as keratinocyte cancers, but they are not synonyms due to the fact that the concept of NMSC also includes other types of skin cancer such as atypical fibroxanthoma, dermatofibrosarcoma protuberans, Kaposis sarcoma or angiosarcoma which are not of keratinocytic origin [10]. Therefore, the term NMSC will be used in this thesis.

BCC is by far the most common type of cancer in Sweden with more than 45,000 histopathologically confirmed cases per year [9]. Calculations suggests that one out of 6 to 7 persons in the Swedish population will develop a BCC by the time they reach 75 years of age [7].

In 2015, 7,063 new cases of NMSC (excluding BCC) were diagnosed in Sweden amounting to approximately 11 % of the total number of cancer cases that year [9]. The great majority of these cases were SCCs, which is the second most common cancer type (excluding BCC) in both men and women in Sweden [9]. However, the exact incidence of the rarer types of NMSC are generally unknown.

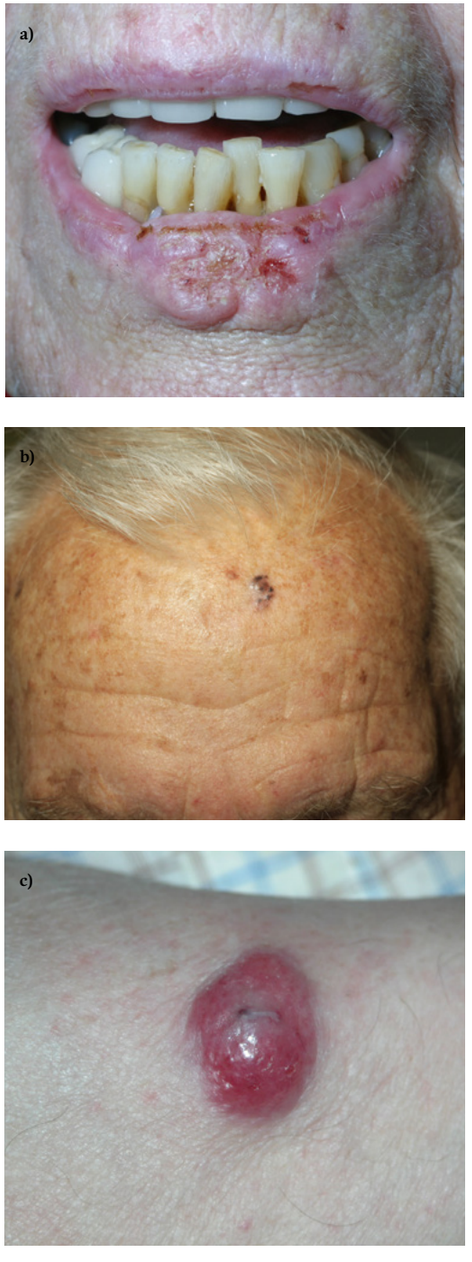


FIGURE 2. Clinical pictures off a) squamous cell carcinoma, b) basal cell carcinoma and c) Merkel cell carcinoma. Photos by John Paoli.

The burden of NMSC leads to high societal costs secondary to treating the tumours [11-13]. *Figure 3* shows the direct health costs annually in different countries for MM and NMSC. In Sweden, the total cost for NMSCs and actinic keratosis (AK) in 2011 (including indirect costs such as loss of production due to morbidity) was 61.2 million euros, which was an increase of around 14 % compared to 2005 adjusted for inflation [13].

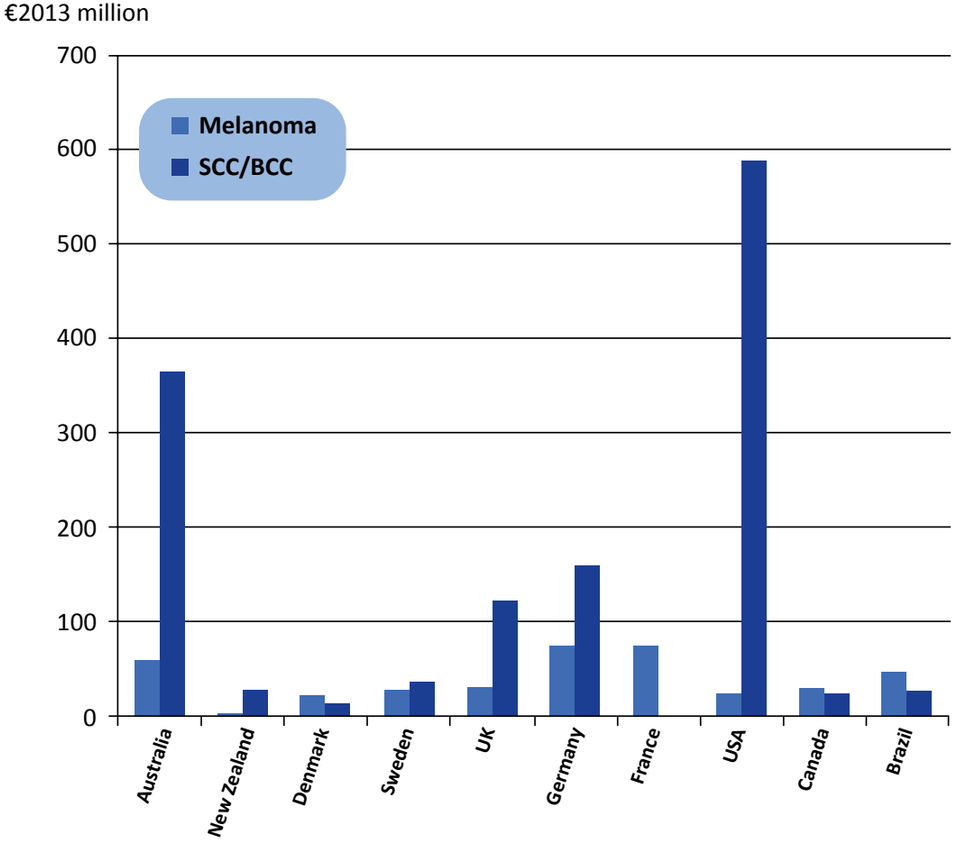


FIGURE 3. Direct health care costs due to the burden of skin cancer each year (SCC, squamous cell carcinoma; BCC, basal cell carcinoma). SCC/BCC make out the majority of NMSCs.

Reprinted from Gordon et. al. Health system costs of skin cancer and cost-effectiveness of skin cancer prevention and screening: a systematic review. Eur J Cancer Prev. 2015 Mar;24(2):141-9. With permission from Wolters Kluwer Health.

1.2.2 Aetiology

The risk for developing NMSC can be contributed to environmental factors and host factors. The major risk factor is UVR exposure and was described in sailors as early as in 1894 [14, 15]. Increasing sun exposure due to traveling, vacations in sunny countries [16] and an active lifestyle are believed to be important factors in the increasing incidence of skin cancer [17].

Another big risk factor for NMSC development is the patient's skin type. Six different skin types were described by Fitzpatrick *(Table 1)* [18]. Individuals with a light skin type who burn easily and tan poorly are more susceptible to the development of NMSCs than individuals with a darker skin type [18].

TABLE 1. Fitzpatrick skin phototypes with corresponding features.

Skin type	Typical features	Tanning ability
I	Pale white skin, blue/green eyes, red/ blonde hair	Always burns, does not tan
II	Fair skin, blue eyes	Burns easily, tans poorly
III	Darker but still white skin	Tans after initial burn
IV	Light brown skin	Burns minimally, tans easily
V	Brown skin	Rarely burns, tans dark with ease
VI	Dark brown or black skin	Never burns, always tans

The incidence of NMSCs in non-Caucasians is 50 times lower than in a population with fair skin [19]. Other factors indicating a person's total UV-exposure and increasing risk of NMSC development are: male gender, increasing age and previous precancerous lesions such as AKs or Bowen´s disease (BD) [10]. Immunosuppressed patients, particularly following organ transplantation, are also at risk of developing NMSC, and have up to 100-250 times higher probability of acquiring

a SCC [20, 21]. Additional but less common risk factors include previous PUVA (psoralen and UV-A) treatment, arsenic intake, ionizing radiation as well as genetic disorders (e.g. albinism, xeroderma pigmentosum and epidermodysplasia verruciformis) [22]. Smoking as an individual risk factor has been shown to increase the risk of SCC but not BCC and the strongest relationship is observed in SCC of the lip [23].

1.2.3 Diagnosis and imaging

The diagnosis of skin cancer is often done by combining a visual examination of the patient with the use of dermoscopy and histopathology. However, this is a field where several new non-invasive techniques are emerging as a complement to the abovementioned methods and the most relevant ones are also explained in this chapter.

Visual examination

The first and simplest examination a dermatologist does when assessing a patient with a skin lesion of concern of any sort is a visual examination. One study found that the sensitivity and specificity of a visual examination of an experienced dermatologist was 93.3% and 97.8%, respectively, when carrying out full body skin examinations in random patients with any kind of skin lesions using only the naked eye and unassisted by other tools. However, the positive predictive value was only 54.0%, meaning that lesions with a suspicion of skin cancer only turned out to be one in a little more than half of the cases. Meanwhile, the negative predictive value was 99.8% [24]. In UK, family practitioners have been reported to miss one third of skin cancers when relying solely on visual examination [25]. In another study comparing dermatologists and family practitioners assessing NMSCs, only 22% of the histopathologically confirmed cases were diagnosed correctly by family practitioners compared to 87% by dermatologists [26].

Dermoscopy

An important tool for increasing the chances of detecting skin cancer is dermoscopy. It is a non-invasive technique consisting of a magnification lens, illumination through light-emitting diodes (LEDs) and, in most of the products on the market, also a polarising filter, which reduces glare from the skin. With a polarising filter, the need for direct skin contact is eliminated which allows for a quicker examination. Dermoscopy in NMSCs was not studied as much compared to pigmented lesions in the early days of dermoscopy. However, there are now several studies available describing algorithms and dermoscopic findings for NMSCs and non-pigmented skin lesions [27]. Studies have shown favourable diagnostic accuracy for NMSCs, e.g. the sensitivity for BCC ranges from 87% to 96% and the specificity from 72% to 92% [28]. A recent publication added more knowledge to the subject by confirming the value of dermoscopy in NMSCs but stressing the value of training in dermoscopy since experts performed much better compared to novel users [29]. Adding dermoscopy to a full body skin examination isn't as time-consuming as could be expected with an examination requiring 70 seconds without dermoscopy and 142 seconds with dermoscopy. This is an acceptable time addition considering the increased sensitivity and specificity it provides [30].

FIGURE 4. Different types of handheld dermatoscopes. Photos by Morgan Carlsson.

Optical coherence tomography (OCT)

OCT provides a vertical cross-sectional view of skin by utilising back-reflectance of light with a wavelength of 1300 nm, which penetrates down to 1-2 mm depth [31]. Recent studies on its use in NMSC have showed a diagnostic accuracy of 87.4% [32], but the resolution at 7.5 μm is not at the cellular level. Apart from diagnosing different skin tumours, OCT can also be used in determining tumour delineation and thickness before surgery. A recent development in this field is dynamic OCT (D-OCT) which utilises rapidly repeating scans of OCT and compares changed and unchanged regions between two scans. This technique can conjure up an image of the vascular structures of e.g. a BCC to a depth of 1.5-2 mm which is deeper than what is possible with reflectance confocal microscopy (described below). Three-dimensional visualization of vascular networks is also possible via special software [31]. Lately, high-definition OCT (HD-OCT) has been introduced enabling even higher resolution at 3 μm [33].

Reflectance confocal microscopy (RCM)
RCM is also a non-invasive diagnostic technique that uses a laser of 830 nm and a pinhole between the detector and the tissue being analysed to obtain en-face optic sectioning of the epidermis and superficial dermis. The pinhole rejects scattered out-of-focus light so that only in-focus light from the tissue reaches the detector. The technical maximum depth is around 350 µm but in clinical practice the maximum depth is closer to 200 µm, which is a limitation when imaging thicker tumours [34]. The digital images obtained with RCM have a resolution close to that of a histological slide (lateral resolution of 0.5-1 µm and axial resolution of 3-4 µm), but from a horizontally sectioned view compared to the classic vertical sectioning done in histopathology. The technique is user-dependent and requires experience before you can reach the same diagnostic accuracy in NMSCs as reported in studies with sensitivity values of up to 94% or even 100 % [28]. Lastly, this method is time consuming [34].

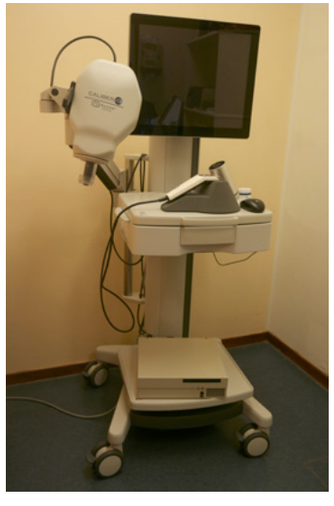


FIGURE 5. A reflectance confocal microscope at Sahlgrenska University Hospital. Photo by Morgan Carlsson.

Multiphoton laser scanning microscopy (MPLSM)
In vivo MPLSM uses a titanium-sapphire femtosecond pulsed laser with wavelength of 710-920 nm to perform optical sectioning of the outer layers of the skin (maximum depth 200 µm) producing horizontal pictures of the skin with a higher resolution (lateral resolution <2 µm vertical and axial resolution <0.5 µm) compared to RCM. Contrast mechanisms rely on optical characteristics which are intrinsic to the tissue and not from using fluorescence [35]. In regards to its use in the diagnosis of NMSC, a recent study on BCC showed promising results, but the number of included patients was small and no reliable diagnostic accuracy could be calculated from this [36].

Raman spectroscopy (RS)
RS works via a near infrared laser with a wavelength of 785-830 nm that excites different molecules in tissue which then emits vibrational energies [37, 38]. Such vibrational energies as well as biochemical changes in the tissue can be measured via a spectrometer and a so-called Raman probe. Collected data can be analysed to discriminate between NMSC and healthy tissue [38]. This technique is based on point measurements and does not provide an image. The majority of studies using RS have been carried out in vitro, but in recent years, more and more in vivo studies have been published. NMSCs can be discriminated from normal skin using RS with an accuracy comparable to that of dermoscopy (85 % in SCCs and 73% in BCCs) [39].

High Resolution Ultrasound (HRUS)
Ultrasound has undergone refinement in the last decades and there are now high-resolution versions commercially available. Resolutions of up to 60 x 200 µm and penetration depths of 23 mm are feasible through the use of a 20 MHz transducer [40]. The technique has been used for measuring tumour dimensions and for tumour delineation, but also for detecting residual BCC after non-surgical treatments with mixed results [40-42].

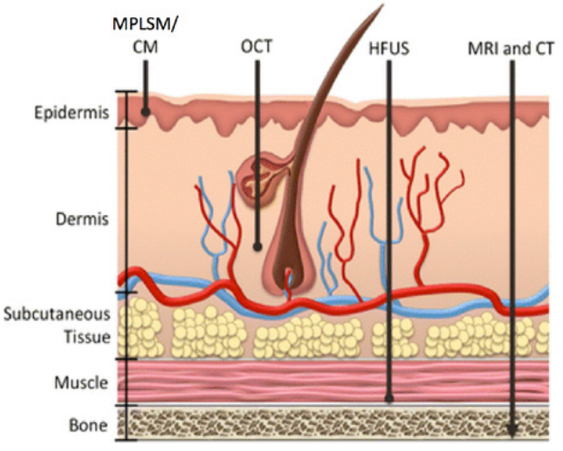


FIGURE 6. A schematic image visualising the imaging depth of different diagnostic skin imaging modalities compared to the total body penetration of magnetic resonace imaging (MRI) and computer tomography (CT). The higher resolution achieved with Multiphoton laser scanning microscopy (MPLSM) and Confocal microscopy (CM) yields lower imaging depth and vice versa. The imaging depths for Optical coherence tomography (OCT) and High Frequency/Resolution Ultrasound (HFUS) is also shown. Edited from Olubukola Babalola, Andrew Mamalis, Hadar Lev-Tov et al. Arch Dermatol Res. 2014 Jan;306(1):1-9. With permission from Springer Nature.

1.2.4 Treatments
There is a wide range of methods available to treat NMSCs, including topical drugs, destructive techniques, photodynamic therapy (PDT), and surgical treatment. These methods and their indications are summarized in *Table 2*.

TABLE 2. Indications and effectiveness of the most common treatments for NMSC. C&E, curettage and electrodesiccation; Cryo, cryosurgery; Laser, ablative laser; PDT, photodynamic therapy; Imiq, imiquimod; 5-FU, topical 5% 5-fluorouracil; Rx, radiotherapy; AK, actinic keratosis; SCCis, SCC in situ/Bowen's disease; sBCC, superficial BCC; nBCC, nodular BCC; iBCC, infiltrating BCC; aBCC, aggressive or sclerosing BCC; 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. Reprinted and edited from John Paoli's thesis "Selected aspects on improving the management of skin cancer" (University of Gothenburg, 2009) with permission from the author.

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

1.2.4.1 Topical therapies

Imiquimod, which is available in 3.75 % and 5 % concentrations, is an immune-response modulator stimulating toll-like receptor 7. It has been licensed for treating AKs and superficial BCC, though in different regimes. Efficacy in AKs with 50% complete clearance rates have been reported in a meta-analysis [43]. For superficial BCCs, daily application of imiquimod for 6 weeks had high initial and sustained clearance rates after 5 years of 94.1 % and 85.4 %, respectively [44]. Off-label treatment has also been tested for BD and also lentigo maligna, an in-situ stage of MM on chronically sun-exposed body parts.

5-Fluorouracil (5-FU), inhibits DNA synthesis by blocking the enzyme thymidylate synthetase [45]. Topical 5% 5-FU cream is used for the treatment of AK, superficial BCC and BD, but it is not commercially available in Sweden anymore. However, it can be prescribed via special license applications to the Swedish Medical Products Agency. A Cochrane review ranked 5% 5-FU as the topical treatment with best evidence for treating AKs but a lot of the studies had methodological difficulties with randomisation, histopathological confirmation and different efficacy outcome definitions. [46]. Open trials have demonstrated around 70 % clearance of multiple AKs [47]. Recently, a new formulation combining 5-FU (0.5 %) and salicylic acid (10 %) was released commercially with promising results for single AKs [48]. For superficial BCCs, studies using 5 % 5-FU alone show similar clearance rates to those of imiquimod [49]. For BD, 5 % 5-FU was considered to be as effective as PDT but with more adverse effects [50].

Ingenol mebutate is an extract from the sap of the plant Euphorbia peplus (or milk weed in English), which has been shown to be an effective treatment for AKs. Its use for treating AKs was approved by the US Food and Drug Administration (FDA) as well as the European Medicines Agency (EMA) in 2012. Topical gels are available in 0.015 % and 0.05 % concentrations for use on AKs located on the face and trunk, respectively. The exact mechanism of action of ingenol mebutate is still unknown. In one 12-month observation study, the lesion reduction rates were approximately 87 % compared with baseline [51] and complete clearance after 2 months was seen in 42 % of the patients in another study [52]. The use of ingenol mebutate in BD and BCC are considered off-label and the scientific evidence so far is scarce mainly consisting of case reports or small retrospective chart reviews [53, 54].

PDT is discussed in further detail in section 2, but the efficacy of this treatment will be described briefly here. Response rates of PDT on AKs ranges from 69 % to 93 % in different studies and are summarized in the latest AK treatment guidelines from the British Association of Dermatologist and in a recent Cochrane report [46, 47]. Relapse rates of 24 % have been reported after 12 months of follow-up [55].

1.2.4.2 Destructive/ablative therapies

Cryotherapy means using liquid nitrogen at a temperature of -196 °C in a spray gun or a cotton-tipped applicator, to destroy a (pre)-cancerous lesion. Depending on the type of lesion, freezing is performed in different regimes. For example, AKs are usually treated with a single short continuous freeze spray, whereas nodular BCCs are usually treated with two longer freeze-thaw cycles after curettage to debulk the tumour. In BCCs, the clearance rates have been reported to be above 90 % [56]. However, since cryotherapy is user-dependent, experience and robust treatment protocols are needed in the everyday clinical work to ensure efficacy [57]. Cryotherapy has also been reported to be an excellent treatment alternative for BCCs on high-risk areas such as the eyelids [58], ears [59] and nose [60]. Nevertheless, effective cryotherapy may result in hypopigmentation due to destruction of melanocytes and scarring which should be taken into account when choosing treatment [61].

Curettage and electrocautery or electrodesiccation (C&E) entails removing the macroscopic tumour tissue using a semi-sharp curette followed by destruction from thermal energy generated from high frequency electricity in order to also target the microscopic presence of tumour cells [62-64]. For BCCs on the extremities, the reported overall 5-year recurrence rate is 3.3% [65].

1.2.4.3 Surgical treatment

Surgery entails removing a skin lesion of concern or a biopsied and histopathologically confirmed skin cancer with adequate margins of healthy skin using a sharp knife or scalpel with subsequent reconstruction of the skin defect. In dermatological surgery, the most common form of excision is an elliptical excision where the length of the excision classically is three times the width ensuring an optimal primary wound closure. The excised tissue is thereafter sent for histopathological examination for assessing the diagnosis and whether or not the lateral and deep margins are free of tumour. There are several guidelines available with recommendations on excision margins in NMSCs **[66-68]**.

Mohs micrographic surgery (MMS) is the gold standard when treating morphoeic and other highly aggressive subtypes of BCC in Sweden. Internationally, BD, SCC and other NMSCs (e.g. MCC and dermatofibrosarcoma protuberans) are also treated with MMS as a first-hand option. The technique involves intraoperative complete margin control through a step-wise histopathological evaluation of removed tissue until all cancer cells are removed. The clinically visible tumour is excised with a 3-4 mm margin at a 45-degree angle at the lateral margins. Subsequently, the freshly excised tissue is flattened and frozen prior to horizontal sectioning. Thus, the lateral margins are seen at the same plane as the deep margin during the histopathological analysis in a microscope. If any tumour cells are observed, these can be mapped and another stage of MMS can take place with the process repeated until no more cancer cells are detected. Finally, the wound is closed and the patient goes home. All this can usually be done in a single day. In Sweden, MMS is the preferred option on highly aggressive BCCs in the facial region with 5-year recurrences rates of 2.1% for primary BCCs and 5,2 % for recurrent BCCs in a Swedish cohort [69].

1.2.4.4 Systemic therapy

Systemic therapy with hedgehog (Hh) inhibitors including vismodegib and sonidegib, which both have received FDA and EMA approval, in inoperable BCCs is a promising emerging possibility. Hh inhibitors are suitable for patients with Gorlin's syndrome and patients with locally advanced or metastatic BCCs that are unsuitable for surgical treatment or radiotherapy. Vismodegib has demonstrated response rates of up to 68.5 % in patients with locally advanced BCCs (laBCC) and 36.9 % in patients with metastatic BCCs (mBCC) [70]. In patients with Gorlins's syndrome, a study showed that vismodegib inhibits growth of new BCCs and diminishes the tumour burden of existing BCCs. However, more than half of the patients had to discontinue the treatment due to side effects [71]. Sonidegib allows for response rates of up

to 71.2 % in laBCCs and 23.1 % in mBCCs, but the drug is not approved for mBCCs in Europe [72]. However, there are also disadvantages of these Hh inhibitors including drug resistance and adverse events with the most common being: ageusia, muscle spasms, alopecia, weight loss or even the occurrence of other cutaneous neoplasias such as SCCs [73].

1.2.5 Prevention

Prevention of NMSC can be discussed in terms of primary, secondary and tertiary prevention. Primary prevention focuses on limiting exposure to risk factors for the development of NMSC with the ultimate goal of avoiding their appearance. This means reducing the exposure to imprudent amounts of UVR from the sun. Wearing appropriate clothing when outdoors and sunscreen use is another form of primary prevention. Several primary prevention campaigns have been carried out around the world with varying grades of success and have been assessed by the United States Preventive Services Task Force [74]. One good example of a well-coordinated effort is the Australian Sun Smart Programme, which targets young students in primary and middle school in Australia, teaching sun safety and helping schools provide safe sun zones with adequate shade, for example. Calculations have shown that every invested dollar in the Sun Smart Programme would yield a return of 130% in terms of less societal costs for skin cancer development and treatment [75]. Another strategy is to affect the general population's opinion on tanning. Different countries and cultures regard the "optimal tan" in different manners. A study from 2010 by Brännström et al. showed that Swedes have the most extreme view of what is considered to be the ideal tan (i.e. they preferred the most tanned image in *Figure 7*) in an international survey [76].

FIGURE 7. Computer-generated photos of people with different levels of tanning. Participants from Sweden had the highest preferred level of tan of all participating countries, choosing the photo in the bottom left corner in general.

Reprinted from Brännstrom et. al. Melanoma risk factors, perceived threat and intentional tanning: an international online survey. Eur J Cancer Prev. 2010 May; 19(3): 216–226. With permission from the author.

Sunscreen use for the prevention of NMSC development is a controversial subject. It has been difficult to design a study with a solid and reliable methodology including enough follow-up time to show statistical differences in terms of reduced incidence of NMSC. A common setup in these studies is to compare new NMSCs in a group of people given sunscreen every day with those occurring in another group allowed discretionary use of sunscreen. Older studies have shown less development of new SCCs but not BCCs with daily sunscreen use [77, 78], but a recent Cochrane review from 2016 couldn't find evidence of beneficial sunscreen use for any type of NMSC [79]. There is a varying grade of methodological problems in sunscreen studies involving how often people apply sunscreen, the thickness of the layer applied, the sun protection factor (SPF), the UVR filters, the lack of histopathological confirmation of the observed new NMSCs, etc. Thus, the conclusions from these studies are hard to interpret in many cases.

In AKs, the scientific evidence for regular sunscreen use, is much more robust. Decreased development of new AKs by regular use of sunscreen was first described by Thompson et al. [80] and these findings have been reproduced several times [81, 82]. Also, the regular use of sunscreen with high SPF in immunosuppressed patients for two years have been reported to reduce both the number of AKs and SCCs significantly in these patients [83].

Secondary prevention focuses on early detection and treatment of NMSCs. This includes: screening campaigns; easy access to healthcare; "e-healthcare" including teledermoscopy as well as education of patients in order to enhance their ability to self-detect NMSCs early. All these measures aim to find and treat NMSCs as early as possible allowing for a better outcome, both in terms of morbidity and mortality.

Tertiary prevention includes standardised follow-up (FU) programs to prevent progression of NMSCs and to ensure the best possible outcome for patients who have already been diagnosed with these cancer types.

1.3 Actinic keratosis

In 1896, Dubreulih was the first to describe AKs [84]. Clinically, AKs present as a flat macule or slightly thicker plaque with white or yellow scales (hyperkeratosis) on the surface *(Fig.7)*. The understanding of AKs has developed during the years but much is still valid from the analysis of AKs being "the seed of cancer". Nowadays, AKs are considered the first clinically detectable areas of skin undergoing the process of carcinogenesis, thus acting as a biomarker of high levels of UVR exposure [85, 86]. Skin field cancerization is considered a chronic disease and refers to the presence of early subclinical transformation on photodamaged skin surrounding AKs that are visible by the eye [47]. Despite the relatively low transformation risk for a single AK lesion to develop into SCC, the presence of multiple lesions on a sun-damaged field over several years increases the risk for the development of invasive and potentially metastatic SCCs. Studies have quantified a 3- to 12-fold risk increase for this to occur [86-88]. However, a recent review article on AK treatments performed by the Cochrane institute could not find any significant data on reduction of SCCs as a result of treating AKs [47]. To date, there is not enough evidence to give grounds for a policy of treating all AKs in order to try to stop cancer development [46]. Nevertheless, most international guidelines recommend treating AKs in general [89, 90].

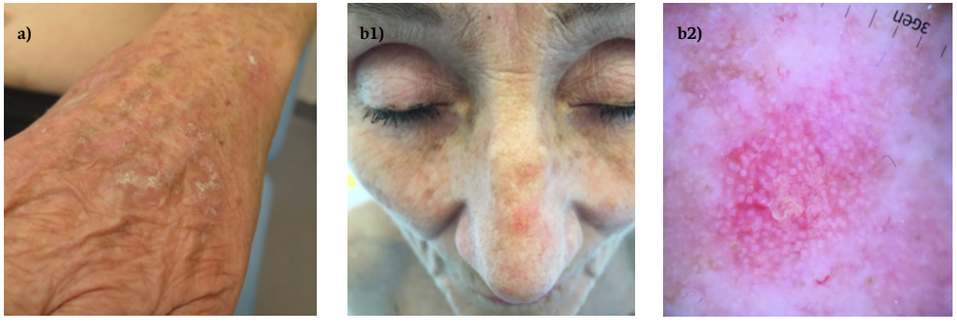


FIGURE 8. a) Clinical and b) dermoscopic images of actinic keratosis. Photos by John Paoli.

1.4 Bowen's disease/SCC in situ

SCC in situ or BD is a premalignant lesion of the skin residing within the epidermis with the capacity to develop into invasive SCC. However, there is a risk of progression from BD in its in situ stage to an invasive SCC, which is approximated to 3-5 % [50, 61]. Clinically, BD presents as a hyperkeratotic, well-demarcated, erythematous plaque with an irregular border frequently occurring on sun-exposed skin of middle-aged to elderly patients *(Fig. 9)*. BD often responds well to treatment and the prognosis for the patient is favourable.

Incidence rates of BD in the scientific literature are scarce. In a Canadian population of mostly Caucasians, age-adjusted incidence of BD was 22.4 and 27.8 per 100,000, in women and men, respectively, with age standardisation to the 1991 Canadian population [91]. Higher incidence rates were reported in Hawaii with 115 per 100,000 in white women and 174 per 100,000 in white men using the 1980 U.S. Caucasian population for age standardisation [92]. Caution should be used when comparing these figures since the studies used different populations when calculating for age standardisation.

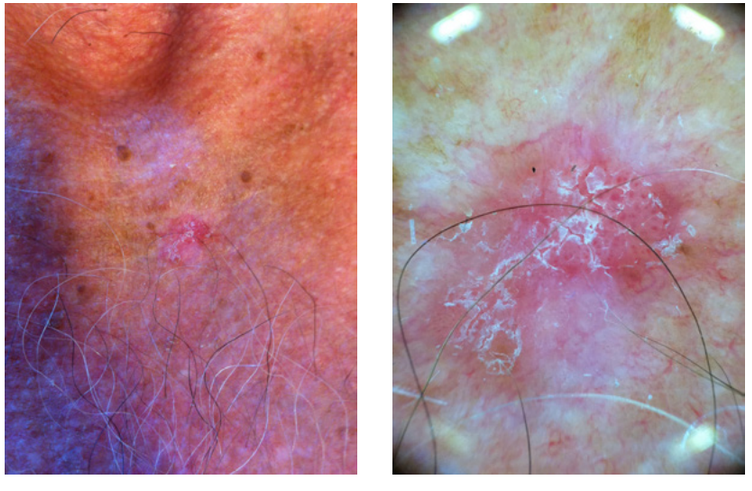


FIGURE 9. Clinical and dermoscopic images of Bowen's disease. Photos by John Paoli.

1.5 Basal cell carcinoma

BCC is by far the most common cancer type in humans [7, 67]. In Europe, reports show BCC incidence rates between 77 and 158 per 100,000 person-years age-standardised to the European standard population [67]. On a local level, the Swedish incidence rates have increased 10-fold over the last 30 years. BCCs are found in the majority of cases in middle-aged to elderly patients on sun exposed body parts and UVR exposure is believed to be the main causative risk factor. [15, 67] BCC is considered to arise from keratinocyte cells located in the dermo-epidermal junction zone and the basal layer, but the exact origin of BCC is still not an established fact [93].

BCCs consist of a group of epithelial tumours that can invade the dermis as well as deeper anatomical structures. Histopathologically, they can be subtyped via their growth pattern on a morphological level. In Sweden, the "Sabbatsbergs" model is used with the categories: Glas type IA & IB, type II and type III [94]. *Figure 10* shows the varied clinical presentations of BCCs according to this classification. Glas types IA & IB are considered to be less aggressive with a minimally invasive nodular or a superficial growth pattern, respectively. Glas type II are moderately aggressive with an infiltrative growth pattern, whereas Glas type III are highly aggressive including the morphoeic or sclerosing subtype.

Nodular BCCs (IA) are the most common group of BCCs and constitute approximately 33 % of all BCCs. They are typically located on the face or trunk and appear clinically as shiny, pearl-like or dome-shaped nodules with arborising vessels and are often partially ulcerated. Superficial BCCs (IB) are mainly found on the trunk and make up for approximately 20 % of all BCCs. Clinically, they can resemble BD and present as hyperkeratotic erythematous plaques, which can also mimic an eczema patch. Infiltrative BCCs (II) comprise approximately 30 % of all BCCs and are mainly found in the facial region. They are often less raised than nodular BCCs and firmer to the touch compared with less aggressive BCCs. Lastly, morphoeic or sclerosing BCCs are also mainly found in the face constituting 5 % of all BCCs. They can resemble a scar or sclerosis and are hard to demarcate clinically. These more aggressive types can infiltrate into other tissues such as muscle, cartilage or bone. BCCs rarely metastasise but can cause considerable morbidity to the affected patient, especially when they grow close to or on the lips, nose, ears and/or eyelids. BCCs with a mixed histopathological growth pattern as well as metatypical or basosquamous variants with differentiation towards SCC are also common [7, 67, 95].

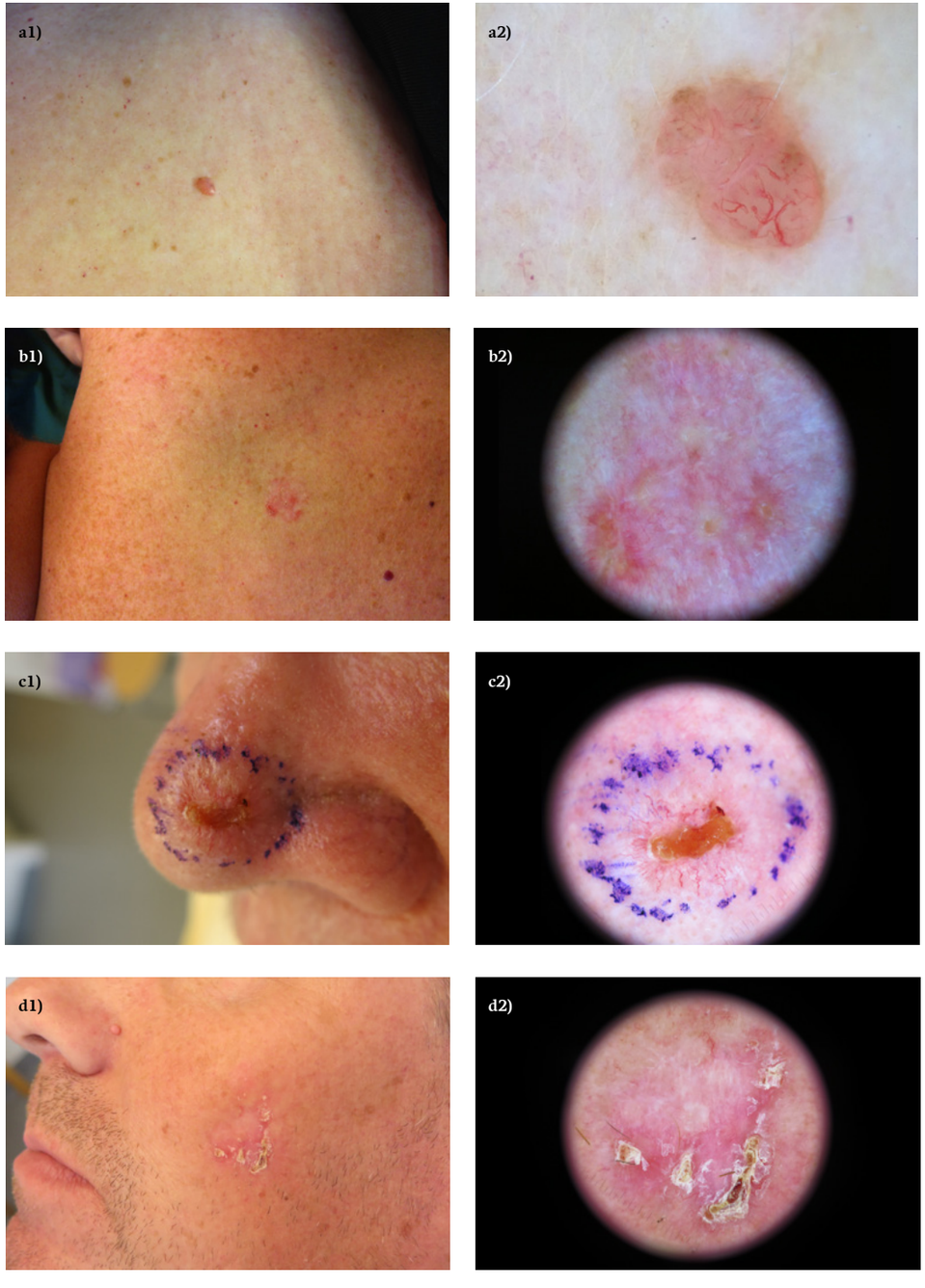


FIGURE 10. Clinical and dermoscopic presentations of the different subtypes of BCC: a) nodular BCC, b) superficial BCC, c) infiltrative BCC and d) morphoeic BCC.

The knowledge of molecular and genetic changes behind the origin of BCC has increased over the latest decades. These insights have partly come from studying patients with different genetic syndromes such as Gorlin's syndrome who have a higher risk of developing BCCs [96]. Individuals with Gorlin's syndrome develop multiple BCCs starting at an early age and the responsible mutation lies in the Hh receptor Patched 1 (PTCH1) gene that mediates Sonic Hh signaling. The PTCH1 genes encodes a protein which functions as a receptor that inhibits the transmembrane protein Smoothened (SMO) when binding to it. When this inhibition is no longer in effect, which is the case with the mutated PTCH gene, several genes involved in controlling cell growth and proliferation are increased [73]. Continuous research has reported atypical activity and mutations in PTCH1 in up to 90 % of BCCs making it a target for drug development as discussed previously [93].

1.6 Merkel cell carcinoma

MCC is a rare type of NMSC, which is considered to be a neuroendocrine tumuor meaning it has both endocrine and epithelial immunohistochemical and histopathological characteristics. Its origin is believed to be the Merkel cell in the epidermis. However, this is not the only theory, and other cells including fibroblasts and skin stems cells have also been proposed as the cell of origin for MCC *(Fig. 11)* [97]. In the case of stem cells and fibroblasts, the Merkel cell polyomavirus (MCPyV) has been suggested to infect them driving the characteristics of the original cell to that of a MCC cell [4]. MCC affects older people on sun-exposed body parts. It is a very aggressive cancer with high recurrence rates despite prompt surgery and/or radiotherapy and also has a high mortality rate with a 3-year survival rate of 67 % and 5-year survival rate of only 41.9 % [98, 99]. The incidence is reported to be increasing in many parts of the world [100-104] and also in Sweden, as shown in Paper I in this thesis [98].

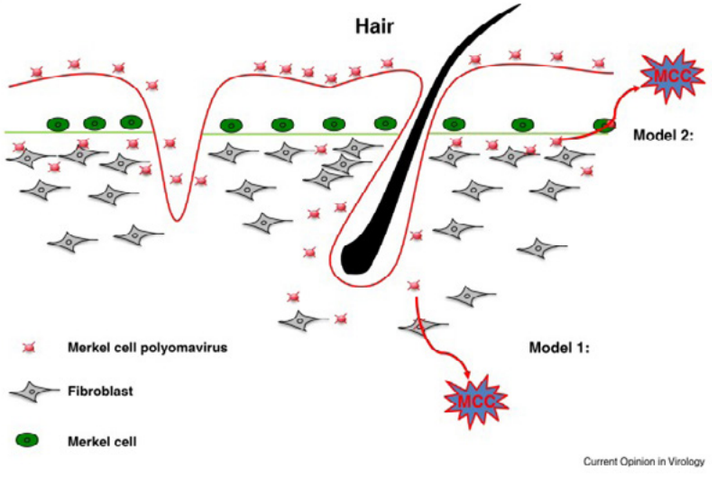
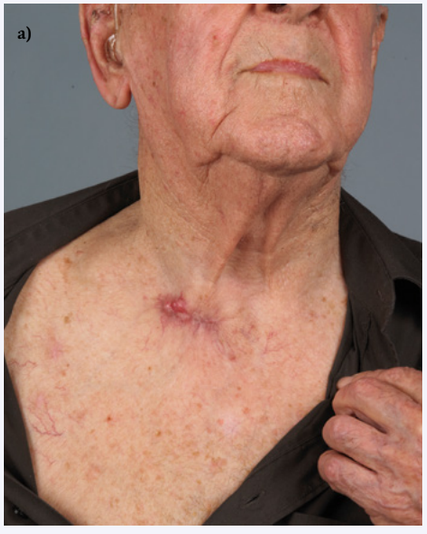
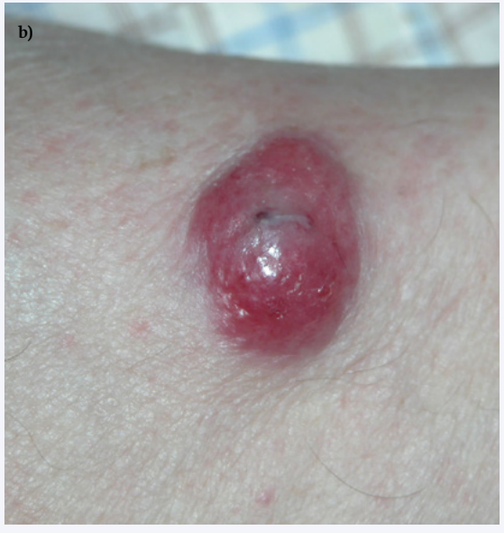
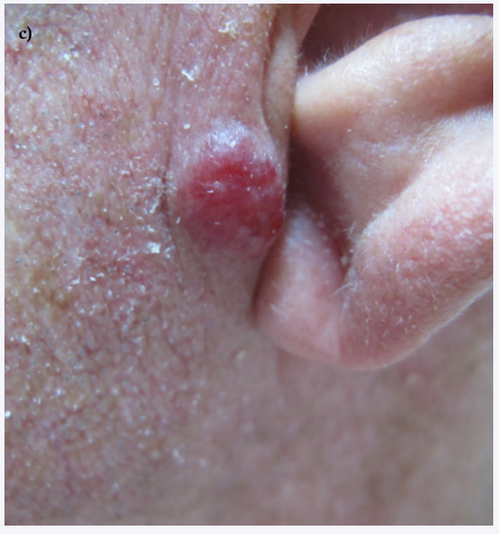


FIGURE 11. Two different models for the interaction between MCPyV infection and MCC development. In human skin, dermal fibroblasts are the foremost target cells of MCPyV infection. In model 1, the dermal fibroblast could be induced by MCPyV infection to transform into MCC. In model 2, progenitor cells or benign Merkel cells located in the proximity of the already infected dermal fibroblasts could be infected as "bystanders". Infection by MCPyV in Merkel cells may lead to MCC.







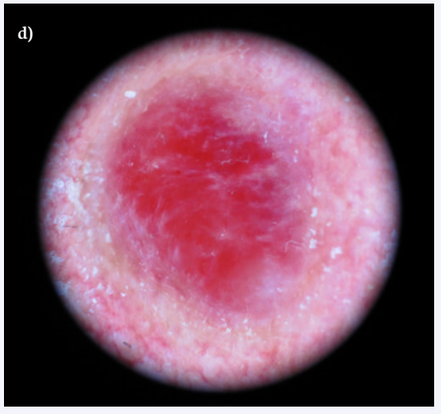


FIGURE 12. Clinical (a-c) and dermoscopic (d) images of Merkel cell carcinoma. Photos by Morgan Carlsson (a), John Paoli (b) and Eva Backman (c-d).

There are several known risk factors for MCC including cumulative UVR exposure, Caucasian skin type, immunosuppression (e.g. in the context of lymphoproliferative illness, organ transplant recipients or HIV/AIDS) and high age [98, 104-106]. MCC can also be classified according to their positive or negative association to MCPyV infection. MCPyV-positive MCCs constitute approximately 80 % of all MCCs. The MCPyV was first described by Feng et al. in 2008 and is considered to be an important factor in driving the oncogenesis in MCC, although the exact mechanism is still unknown [107]. MCPyV-negative MCCs are found more often in countries such as Australia with very high levels of sun exposure with oncogenesis driven more by UVR [106]. MCPyV-negative MCCs also have a less favourable prognosis [108, 109].

Clinically, MCCs are easily missed. They often present on sun-exposed areas as a firm, painless, pink or violaceous nodule with rapid growth and can be misinterpreted as another type of NMSC, a benign tumour, a cyst, a chalazion or even a wart depending on the body part *(Fig. 12)*. To overcome this, the acronym AEIOU was proposed by Heath et al. [105]. It stands for:

A: Asymptomatic – 88 % of MCCs in the study were not tender to touch and did not cause the patient any discomfort at the time of diagnosis.
E: Expanding – most MCCs appear in 3 months or less which differentiates MCC from a BCC for example.
I: Immunosuppression – it is 16 times more likely that a person with MCC is immunosuppressed as compared to the US standard population.
O: Older age – close to 90 % of MCC patients are over 50 years of age.
U: UVR exposure – the majority of MCC lesions are located on sun-exposed skin.

1.6.1 Epidemiology

Hans Rosling, the famous Swedish professor of epidemiology and public health, once said "As our world continues to generate unimaginable amounts of data, more data lead to more correlations, and more correlations can lead to more discoveries". It is one of the most popular quotes on the internet regarding epidemiology, which is the branch within medical research that deals with incidence, distribution of illnesses and other health-related states. It is of great importance to half of the articles in this thesis ("Merkel cell carcinoma incidence is increasing in Sweden" and "Effectiveness of photodynamic therapy in Bowen's disease: a retrospective observational study in 423 lesions").

Hippocrates is thought to be the initiator of epidemiology with his thinking on how environmental factors influence illness. In fact, the term derives from the greek words 'epi' meaning upon, 'demos' meaning people, and logos, meaning the study of, roughly translating into "the study of what is upon the people". Modern epidemiology started much later, approximately 150 years ago with the famous findings of John Snow concerning the cholera epidemic in London during the 1850s [110]. He could elegantly show that deaths in cholera were correlated to where people got their drinking water and also formulated the hypothesis that cholera was spread via water. Improvements in water handling and supply were made and cholera death rates dropped significantly. This was done long before the actual microorganism responsible for the disease was discovered but his findings and theories were correct [111].

The scientific thinking of John Snow lives on and epidemiology now uses quantitative methods to analyse illness and health-related problems, for instance, in order to increase awareness of actual facts. An example is Hans Rosling's GapMinder project which prove our ideas based on "common sense"

to be wrong through data analysis (e.g. the idea that more people die from overweight than from starvation in the world or that middle income countries fare worse than both low- and high-income countries in regards to dental health) [112]. Other examples of modern epidemiology are the mapping of an outbreak of avian flu [113] or reporting on the effectiveness of a treatment modality.

In this thesis, epidemiology has been used to calculate incidence, both crude and age-standardised. Crude incidence is calculated by dividing the number of cases with the corresponding population per time unit (e.g. the number of cases of MCC occurring in Sweden during a particular year) and is usually expressed as the number of cases per 100,000 inhabitants per year. An even better way of describing incidence is via age-standardisation in which you take into account the composition of the population where the disease is studied. In order to do this, you need a standard population. Several international standards are commonly used in the scientific community including the distribution of the average world population between the years 2000 and 2025 [114], the US population in the year 2000 [115], the European population in 2013 [116] or the Swedish population from 2000 [9]. When reading incidence figures, it is important to bear these facts in mind. A crude incidence is less exact compared to an age-standardised incidence rate and the population to which the age-standardisation is performed matters[114]. Therefore, when comparing incidence rates observed in various studies, differences in incidence rates can in some cases simply be explained by which population was used for age-standardisation.

Epidemiological methods were also applied in Paper II when calculating the efficacy of PDT for BD as well as to discern risk factors for unsuccessful treatment or recurrence of BD.

1.7 Photodynamic therapy

PDT was first described in the beginning of the 20th century using daylight and acridine orange for the study of Paramecium caudatum cells, and has since then gone through some modifications, both in terms of light sources (though daylight is now back in fashion again) and in photosensitizers, to the modalities we use today [117]. In the 1990s, Kennedy et al. described the use of artificial light in conjunction with the topical precursor to protoporphyrin IX (PpIX), 5-aminolevulinic acid (ALA), and the use of this technique soon started to spread among dermatologists around the world [118].

In daily practice, PDT is widely used in the treatment of multiple AKs or field cancerization, BD as well as BCCs of the superficial (and to a lesser extent of the nodular) type. It is not effective for BCCs that grow deeper into the dermis, for SCCs or more aggressive tumours such as MCC. PDT can be used for photorejuvenation and is sometimes even used to treat conditions such as: extramammary Paget's disease, palmar and plantar warts, infections (e.g. leishmaniasis and mycoses), cutaneous T-cell lymphoma, acne and other inflammatory diseases like localised scleroderma or lichen sclerosus [119-121].

Simply put, PDT requires the presence of three elements simultaneously: a photosensitiser, light energy and oxygen inside the diseased tissue. Today, the most common PDT methods are conventional PDT and daylight PDT [89].

Conventional PDT

Conventional PDT involves two steps. First, a prodrug such as ALA or its methylated ester, methyl aminolevulinate (MAL), is administered on the affected skin. ALA/MAL enters the heme cycle in the affected cells leading to the endogenous production of the photosensitiser PpIX, a process which is intensified in neoplastic tissue compared with healthy tissue. When sufficient PpIX has accumulated in the target cells (usually after 3 hours), it is time for step two, when the affected area is irradiated with a regulated dose of an appropriate light for the absorption spectrum of PpIX *(Fig. 12)* [122].

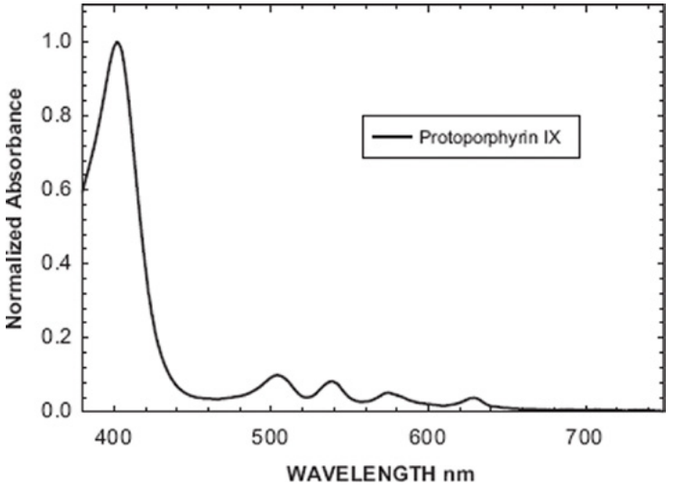


FIGURE 13. Absorption spectrum of protoporphyrin IX. The spectrum peaks at around 400 nm. Four additional smaller peaks are visible starting at slightly over 500 nm with the last peak at approximately 630 nm.

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In Europe, visible red light (≈630 nm) via LEDs is the most commonly used light source. There are however other light sources that can be used including: metal halide lamps, lasers, fluorescent lamps and filtered xenon arc lamps [89]. The light photons excite PpIX in the presence of oxygen leading to the formation of cytotoxic singlet oxygen and other reactive oxygen species (ROS). Ultimately, this destroys the affected cells via both necrosis and apoptosis, particularly in the mitochondria, leading to the clearance of the (pre-)cancerous cells [90, 118, 120].

A notable downside in PDT is the amount of pain that might accompany the treatment. ROS are suspected to be the main cause of this pain via direct stimulation of nerve endings and indirectly via inflammatory by-products [123]. The treatment is commonly described as a very unusual burning or stinging sensation [124]. Clinically, the onset starts early during illumination and, as treatment stops, the pain decreases dramatically. Nevertheless, in some cases it can last for 12-24 hours or more after concluding the treatment [125]. The pain is usually measured

using a visual analogue scale (VAS) scale ranging from 0 (no pain) to 10 (worst pain imaginable). Wiegell et al. investigated fluorescence and found that greater accumulation of PpIX was correlated with more pain [126]. Sandberg et al. showed that the pain experienced during PDT is related to the size of the lesion or treated area, i.e patients with field cancerization or multiple AKs have a higher probability of pain than patients with a single lesion. Over the years, different attempts have been made to lower the pain experienced during PDT including: topical gels with tetracaine and morphine [127, 128], cold water spray during illumination [124], shorter incubation periods and modified exposure with blue light [129], hypnosis [130], fractionated PDT [131], peripheral nerve blocks [132, 133], bi-level irradiance protocols [134] and transcutaneous electrical nerve stimulation [135]. Wang et al. recently reviewed different studies on how pain levels were affected by varying light doses and fluence rates during PDT showing that light doses lower than 20 J/cm^2 and fluence rates lower than 50 mW/cm^2 were associated with less pain [123].

Daylight PDT

In recent years, natural daylight, which is a combination of infrared and visible light, has again gained popularity as a light source for PDT. What started out in the beginning of the 20th century as a necessity due to the lack of artificial light has become one of the more common ways of delivering light energy during PDT in Europe. Daylight PDT has comparable efficacy to conventional PDT [136-139], it is less painful and also uses less time and resources from healthcare personnel [123, 138, 139]. Daylight encompasses all the peaks in the spectrum for activation of PpIX but the most effective light dose for daylight PDT has not yet been determined [137]. As an estimate, the number of lux during a two-hour daylight PDT session should be 10,000 lux, but studies have shown effectiveness with levels as low as 2300 lux [140]. Nevertheless, in Sweden, daylight PDT is only possible to perform during warmer and sunnier parts of the year under clear sky conditions. Since nature is not always easy to predict, artificial or simulated daylight PDT (i.e. using indoor lamps mimicking the green and red components of daylight) has been developed and was described in 2015 by Kellner et al. [141]. Further studies on this modality need to be performed, but it could be an efficient technique potentially combining the efficacy and low pain levels of daylight PDT in conjunction with the reproducibility and reliability of an artificial light source.

1.8 Time of Flight – Secondary Ion Mass Spectrometry

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) is a diagnostic method in which gas clusters/projectiles are fired at a specific spot of a tissue sample to analyse its chemical composition. During this bombardment, molecules representing the sample's chemistry in the targeted area are ejected from this spot at the sample surface and then identified with a mass spectrometer. This process is repeated spot by spot, row by row until the whole surface of the tissue sample has been analysed. Ultimately, a chemical map of the sample is generated, where changes in chemistry can be linked to specific areas on the sample surface *(Fig. 14)*. In this manner, chemical changes in different healthy tissue types or anatomical structures can be observed and chemical differences between healthy and cancerous tissue can be studied [142].

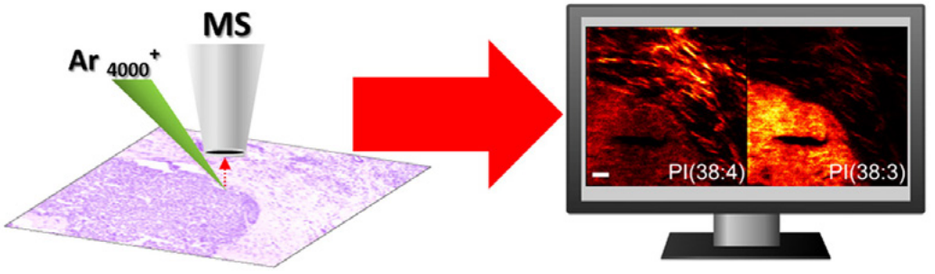


FIGURE 14. Simplified explanation of the chemical map being created in ToF-SIMS.
Reprinted and adapted with permission from Angerer et al. Lipid Heterogeneity Resulting from Fatty Acid Processing in the Human Breast Cancer Microenvironment Identified by GCIB-ToF-SIMS Imaging. Anal Chem. 2016; 88: 11946-54. Copyright American Chemical Society.

There is a growing interest in acquiring a greater understanding of the role of lipid metabolic alterations in oncogenic pathways and how they are connected [143]. Changes in the lipid composition of cells can take place earlier and at a higher rate than changes in proteins and might be the initial sign of a phenotypic modification in cells [144]. ToF-SIMS can help detect lipids in cells and alterations in these cell's lipid metabolism can yield principal information about their metabolic situation, which is hard to explore with regular histopathological methods.

ToF-SIMS enables the study of chemical changes in individual cells by performing precise lipidomics, i.e. describe the complete lipid profile of a specific tissue section, with high resolution. Thus, the chemical composition of a cancerous area in tissue can be mapped and analysed. Lipids in human cancers have been studied before using other mass spectrometry techniques such as desorption electrospray ionization (DESI) and matrix-assisted lased desorption ionization mass spectrometry (MALDI), but because of the low resolution of these techniques, no clear connection to the cancer metabolism could be made [145, 146]. Advances in the ToF-SIMS technique in recent years, particularly the use of gas cluster ion beams (GCIBs), have made it possible to detect lipids with much higher resolution and to elucidate which lipids are connected to cancerous tissue and which are associated with healthy tissue. In regards to skin, reports have been made of significantly elevated levels of phospholipids and total lipids in BCCs in comparison to healthy skin tissue using Folch's method and RS, respectively [147, 148]. Also, phospholipids have been reported to be part of the activation of the Hh pathway described in BCC and Gorlin's syndrome analysed using immunohistochemistry and polymerase chain reaction [149].

2. AIMS

The studies included in this thesis cover the epidemiology, diagnostics and treatment of selected NMSCs. The aims of the investigation were:

- To study the epidemiology and clinical characteristics of MCC in Sweden.
- To evaluate and assess the effectiveness of PDT in the treatment of BD as well as to determine what factors affect the response rates.
- To develop a novel illumination protocol in PDT for AKs making it less painful for the patient but still as effective as the present gold standard.
- To map the chemical composition of lipids in BCCs using ToF-SIMS.

3. Methodological considerations

3.1 Study I

Subjects

The study population consisted of all people living in Sweden during the period from January 1, 1993 to December 31, 2012.

Methods

This was a retrospective cohort study. The Swedish Cancer Registry (SCR) was used to find all patients diagnosed with MCC during the study period. We cross-referenced the SNOMED-2 & SNOMED-3 (a classification system for pathologists) code for MCC (82473), with the ICD-10 code for all invasive NMSCs, ICD C44.0-C44.9. By comparing the two coding nomenclatures we could exclude all other types of NMSC and MCCs found primarily in other organs than the skin. The body location of the MCCs could be calculated using the last number in the ICD-10 code in the following manner: lip '0', eyelid '1', ears '2', other/unspecified parts of the face '3', scalp and neck '4', trunk '5', arms '6', legs '7' and not specified '9'. We aggregated data to simplify the analysis by grouping location codes 0–4 together as 'head and neck' tumours.

Data regarding the TNM classification, which gives information on tumour thickness as well as spreading to lymph nodes and other organs were not available in the SCR for MCC until 2003-2004 and weren't consistently reported until 2005. When TNM data was available, this served as the premise for the following staging of MCC into: stadium I (T1), II (T2-T4), III (any N+ tumour) and IV (any M+ tumour). Worth noticing is the "N"-classification, which the

treating physician registers based on clinical (i.e. palpation of regional lymph nodes) and sometimes histopathological findings. In some cases, a sentinel lymph node biopsy might have been performed, but this was not routinely done in Sweden during the study period. The "N"-classification was therefore often NX, meaning data was not available. Since clinical palpation alone will not detect all spread to the lymph nodes, the number of N+ patients were probably much lower in this cohort than it was in reality. This was also true for the "M"-classification where adequate staging with radiology, including computer tomography and positron emission tomography, was not done routinely during the study period either. This probably lead to a lower number of proven cases with metastasised disease at the time of diagnosis in the SCR compared to the real situation.

Statistical analysis

Fisher´s exact test and logistic regression was used to compare survival and incidence rates over time. The tests were two-sided with a p-value below 0.05 considered statistically significant. Crude and age-standardised incidence rates were also calculated using the European standard population, the US population year 2000 and the world standard population ensuring easy comparison with earlier studies on the subject.

3.2 Study II

Subjects

The study population consisted of all patients who were treated for BD with PDT at Sahlgrenska University Hospital (SUH) during the period of January 1, 2002 to December 31, 2014.

Methods

The study design was retrospective and observational. All electronic patient charts (the electronic patient journal system was implemented at SUH in 2002) with the ICD code D04.0-9 for BD and a specific national treatment code for PDT (DQ004) were selected. They were all manually assessed and only patients with histopathologically verified BD who had at least one FU visit were included in the study. Patients with BD lesions in the anogenital region were not included in the study since these lesions has been proven challenging to treat and have a high risk of recurrence independently of which treatment modality is used as well as a higher risk of transformation to invasive SCC [61, 150]. Thus, including anogenital BD would have affected the observed efficacy rates in a way that doesn't reflect the usage of PDT in routine clinical praxis.

The parameters incorporated in the study were: age, sex, lesion size, body site, histopathological confirmation or not, number of PDT sessions, date of treatment, VAS for assessment of pain during and after treatment, photosensitiser and light source used, clinical outcome at first FU visit, cosmetic result and date of any recurrences detected during following FU visits. Worth noticing was that, in this study, even a finding of an AK in an area treated with PDT was considered as an incomplete response or a recurrence of disease if it was spotted at subsequent FU visits. This is not always the case in similar studies and is important to bear in mind when reading the result section.

Thus, a complete response was defined as no visible sign of dysplasia in the treated area at the first FU visit and a recurrence was defined as any clinical signs or histopathological confirmation of dysplasia at subsequent FU visits. The number of lesions with complete clearance and without any recurrences during any subsequent FU visits divided by the total number of lesions treated with PDT yielded the overall clearance rate.

Statistical analysis

Fisher´s exact test was used on parameters described above to determine whether they were potential risk factors for incomplete response or recurrence. A p-value below 0.05 was considered statistically significant. Kaplan-Meier analysis was applied to visualise treatment success over time graphically.

3.3 Study III

Subjects

Twenty-nine patients were included in a prospective, randomised, two-armed, split-face designed study performed at SUH during September 1, 2015 to March 31, 2017. Previous research has reported VAS to be around 6.0 points on average during PDT with a standard deviation of 2.5 points [132]. Power calculations using Wilcoxon´s signed rank test showed that at least 20 patients were needed in order to reach a power of 0.91 and a significance level of 0.05.

Methods

The study was performed in order to find an alternative illumination protocol for PDT with the goal of offering patients the same effectiveness as the standard protocol but at a lower pain level. Patients older than 18 years with symmetrically distributed AKs were qualified for inclusion in the study. Exclusion criteria were pregnancy or breastfeeding, participation in another study at the same time or possible poor protocol adherence by the patient (e.g. drug or alcohol abuse, severe psychiatric illness, etc.).

All patients received the same ALA photosensitiser but were randomised to begin treatment a) on the left or right side of the treatment area with b) the standard or modified treatment protocol, which resulted in four different scenarios possible for randomisation. The software R version 3.0.3 (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.) was used for randomisation. To ensure equal groups and balance in sample size, an original algorithm was utilised to create a block-randomisation. Before the start of treatment, an envelope was opened revealing which side should be treated first and with which illumination protocol (scenarios A-D). The normal treatment protocol consisted of illumination with an Aktilite CL-128 lamp (PhotoCure ASA, Oslo, Norway) for 8-9 minutes with a total light dose of 37 J/cm^2. The modified illumination protocol utilised a newer lamp called RhodoLED (Biofrontera Bioscience GmbH, Leverkusen, Germany), which has the possibility to gradually increase light intensity during treatment. Both lamps use approximately the same wavelength (630 nm for Aktilite and 635 nm for RhodoLED). A protocol with initial low intensity (20% of maximum for 4 minutes) followed by a period of medium intensity (40 % for 4 minutes followed by 60 % for 12 minutes and 40 seconds) was developed in order to reach the same total light dose of 37 J/cm^2 but during a longer period of time (20 minutes and 40 seconds). Previous research has shown that a fluence rate below 50 mW/cm^2 yields lower levels of pain [126, 151]. The RhodLED lamp has a fluence rate of 77 mW/cm^2 +/- 15 % so 60 % of that as used as the maximum intensity in the study resulted in a fluence rate of approximately 46.2 mW/cm^2 (i.e. below the previous described threshold for pain at 50 mW/cm^2). Apart from the choice of fluence rates in this protocol, a longer illumination protocol was not considered feasible from a practical point of view.

FIGURE 15. The two different lamps used in paper III: Aktilite (a) and RhodoLED (b).

The two PDT sessions with the standard and modified protocols were carried out directly after each other. Pain was assessed using VAS every third minute during each treatment protocol resulting in 2-3 VAS scores for the standard protocol and 6-7 VAS scores for the modified protocol. These VAS scores were used to observe potential pain changes during treatment. After each protocol, the patient was also asked to assess an overall pain score for each treatment side. The overall pain scores were used to compare the pain levels between protocols. The study participants had to complete a FU diary in which they were asked to assess pain, redness, scaling and hyperpigmentation after 1, 2, 7, 14 and 28 days using a simple 4-point scale (0, none; 1, slight; 2, moderate and 3, severe).

The efficacy of the two treatments was calculated by counting the number of AKs in each treatment field before treatment and at the FU visit after 3 months. The dermatologist who performed these evaluations was blinded to which of the illumination protocols had been used on either side.

Statistical analysis

All statistical tests were two-sided and $P<0.05$ was considered as statistically significant. Wilcoxon signed ranked test was used primarily since this is a good test for non-parametric data when comparing two dependent samples that are selected from populations with the same distribution which is the case with the matched study groups in this study.

3.4 Study IV

Subjects

Nine patients with histopathologically verified aggressive BCCs were included in the study. One patient with a less aggressive BCC was added for comparison.

Method

Fresh frozen tissue samples were collected via routine MMS procedures (described earlier in section 1.2.4.3) performed at SUH. After the standard sectioning in MMS was performed, a couple of extra consecutive tissue sections were obtained. The first one was mounted on a conductive indium tin oxide (ITO) slide and the second was stained with hematoxylin and eosin (H&E) prior to being mounted on a normal slide. This extra sectioning did not cause the patient any discomfort, harm or delay in the surgical procedure, which took place as normal.

The mounted frozen tissue was then transported on dry ice to the department of Chemistry and Molecular Biology at the University of Gothenburg where ToF-SIMS analysis was performed. A J105-3D Chemical Imager (Ionoptika Ltd, UK) ToF-SIMS instrument was used for running the analysis *(Fig. 16)*. It is a powerful ToF-SIMS instrument, which only exists in a few similar setups in the world and is described more in detail in earlier works [152].

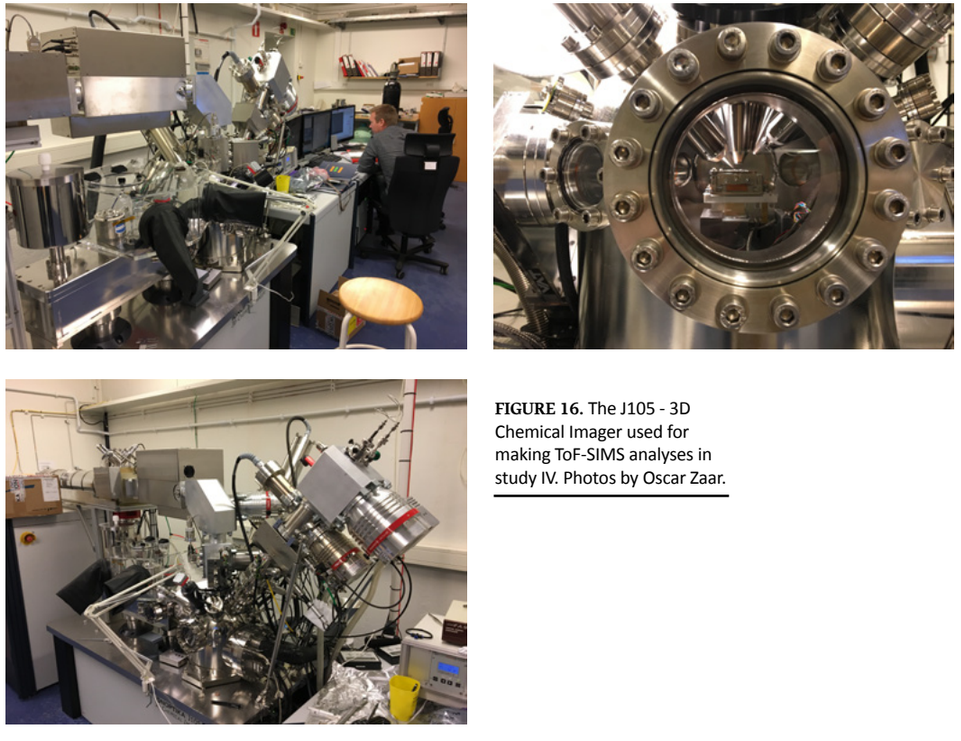


FIGURE 16. The J105 - 3D Chemical Imager used for making ToF-SIMS analyses in study IV. Photos by Oscar Zaar.

A layman explanation of using the J105-3D Chemical Imager on the tissue samples mounted on the ITO slides, is that it provides chemical spectrums of a single spot. This is shown as a diagram like the one in *figure 17*. The m/z ratio on the horizontal axis in a mass spectrum means mass divided by charge number.

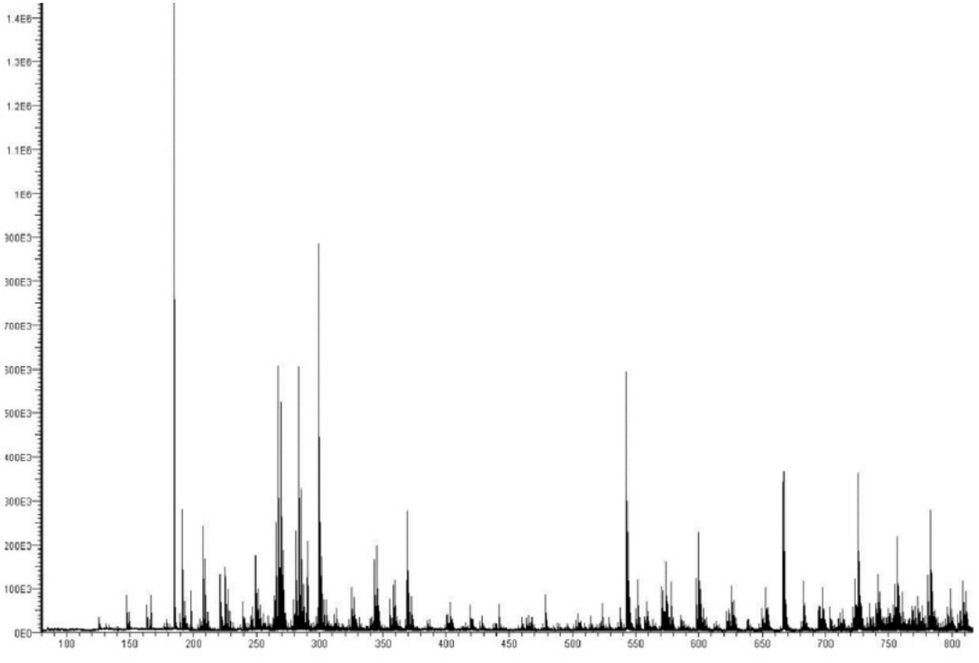


FIGURE 17. Peaks from total positive ion mode in ToF-SIMS of a BCC. The x-axis shows mass (m/z). These data are later processed in MAF to better visualise differences between healthy and cancerous regions.

A single spot of tissue can have several different peaks in its spectrum. These peaks can be "translated" into lipids by consulting lipid map databases. For instance, a peak at 369.35 m/z can be classified as a [M+H-H2O]+ ion of cholesterol. Analyses are made in both positive and negative ion mode revealing different lipids. In addition to this, Maximum Autocorrelation Factor (MAF) was used. This is a mathematical tool for finding repeating patterns and is a form of data reduction technique described in further detail by Henderson et al. [153]. Different MAF scores can then be displayed as a map over the tissue using different colours to locate positive and negative scoring pixels (e.g. green for positive scoring pixels and red for negative ones) (*Fig.18* and also *Fig.3* on page 3 in paper IV).

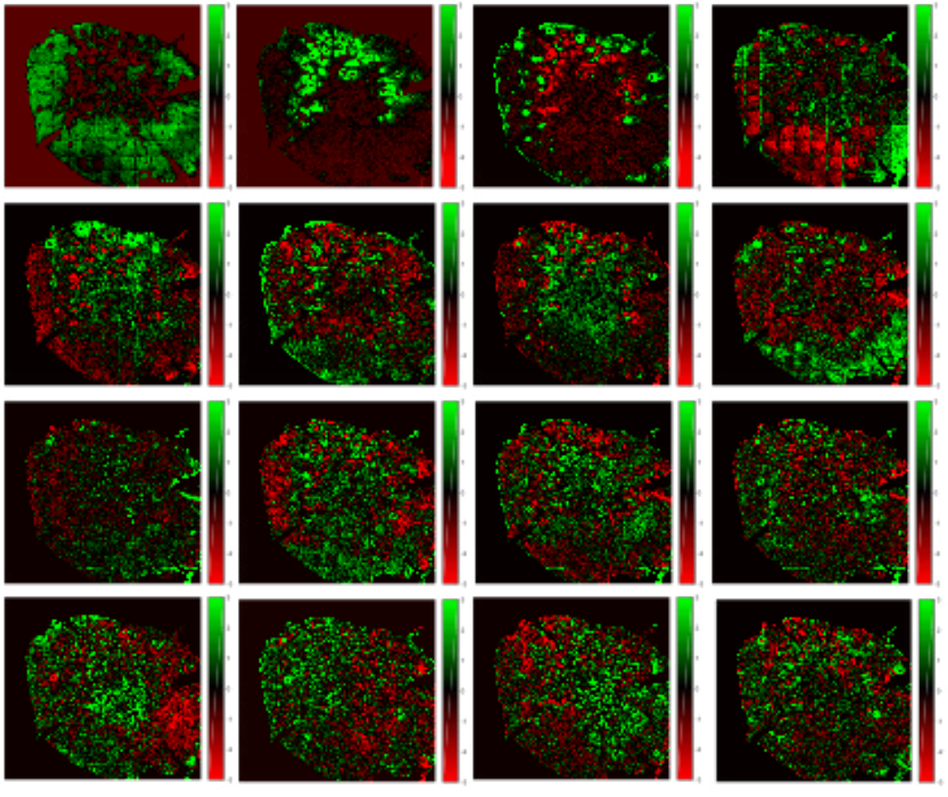


FIGURE 18. MAF score images in which green indicates positive scoring pixels and red stands for negative ones. Each MAF score image, highlights a difference in peaks seen in total ion mode in ToF-SIMS.

Statistical analysis

Study IV differs from the other three included in the thesis in this regard. No statistical analysis was performed as this was a descriptive study. Instead MAF was used to simplify and correlate the data described above.

3.5 Ethical considerations

All four studies included in this thesis received approval from the Regional Ethical Review Board of Gothenburg prior to being carried out. The reference numbers from the Ethical Review Board were: 471-14 for paper I, 941-15 for paper II, 737-15 for paper III and 499-16 for paper IV. In study IV a biobank application was also approved.

4. Results

4.1 Study I

The age-standardised incidence of MCC in Sweden almost doubled during the study period. The overall incidence for women and men per 100,000 persons, using the world population for age standardisation, rose from 0.11 to 0.19 between 1993 and 2012.

Depending on which population was used for standardisation, the increase was 73-85 % whereas the crude incidence rose by 113 %. The affected persons were elderly, with almost all cases (98.9 %) presenting in persons older than 50 years of age and more than half (55.7%) diagnosed in persons older than 80 years. The age-adjusted incidence was slightly higher in men than females, which is consistent with findings in other studies [103, 154]. The majority (51.8 %) of MCC cases was found in the head and neck area.

Persons affected by MCC had a lower overall survival rate when compared to a control group which was matched for sex-, age- and period-specific mortality rates. The 5- and 10-year survival rates for MCC in all stages combined was 41.9 % (95 % CI 36.2-48.5) and 21.4 % (95 % CI 16.8-27.1), respectively, compared to that of the age- and gender-matched population which was 62.3 % and 36.8 %, respectively.

Furthermore, the results of study I show that the majority of MCC were not spread to lymph nodes or other organs at the time of diagnosis. Only 9.3 % of the cases had confirmed spreading to the lymph nodes at the time of diagnosis and just 2.9 % had a known distant metastasis. However, as mentioned in the methodological considerations, staging procedures such as sentinel lymph node biopsy or (PET)-CT were not performed in most cases during the study period. This probably lead to an underreporting of the “N” and “M” stages.

4.2 Study II

During the study period from 2002 to 2014, a total of 423 BD lesions in 335 patients fulfilled all the inclusion criteria. All patients were treated with the same light source, i.e. the Aktilite CL-128 lamp (Photocure ASA, Oslo, Norway), the same MAL photosensitiser (Metvix®, Galderma, Paris, France) and the same total light dose of 37 J/cm^2. The majority of treated patients (63.6 %) were female and elderly with a median age of 79 years. The head and neck area was the most common body part treated with PDT followed by the lower extremities (much more common in women than in men). A quarter of the lesions were larger than 20 mm in diameter.

The complete clearance rate was 63.4 % after a median FU time of 11.2 months (range 0.2-151months). Two risk factors proved to be associated with statistically worse outcome: lesions greater than 20 mm in size and a single session of PDT. For larger lesions, i.e. above 20 mm in diameter, the complete clearance rate was only 48.7 % compared to smaller lesions with 68.7 % (p=0.002). Cases treated with one PDT session had a complete clearance rate of 48.1 % compared to 65.6 % when using two treatment sessions (p=0.016). No other parameters assessed significantly affected the clinical outcome of PDT as shown in *Table 3*.

TABLE 3. Parameters which did not affect complete clearance rate of BD treated with PDT. P<0.05 was considered to be statistically significant.

Parameter	P-value
Age	0.94
Gender	0.46
Time elapsed between treatment sessions (1-4 weeks)	0.27
Body part	0.26

In a little more than half of the cases (59 %), a description of the cosmetic result of PDT could be retrieved from the patient charts. Of these, almost 4 out of 5 lesions (78 %) had healed without any adverse events such as scarring, erythema, hypopigmentation or hyperpigmentation. The most commonly reported adverse event was scarring, which affected 8.8 % of the cases.

4.3 Study III

In total, 399 AKs in 29 patients were treated. The treatment areas were matched for the number of AK lesions with 201 AKs (range 3-13, median 6.0 AKs) in the Aktilite group compared to 198 AKs (range 3-13, median 6.0 AKs) in the RhodoLed group.

The new modified illumination protocol with step-wise increases in the light intensity resulted in a lower overall pain score of 4.5 on the VAS score compared to the standard protocol with 5.8 points. However, when comparing each patient with him or herself, the decrease of the median VAS score was only 1.1 points on the VAS score (0.3-1.7 95 % CI, p=0.0091). A separate analysis was performed on pain during the first nine minutes of treatment (the treatment time of standard illumination protocol with Aktilite) and the difference in experienced pain decreased in a similar manner with 1.3 points (p=0.0018).

When assessing efficacy on individual AKs, the clearance rate at 3 months FU was 93.7 % (89.7-97.8 95 % CI) with Aktilite and 91.2 % with RhodoLED (86.1-96.3 95 % CI). This was a small but statistically significant difference (p=0.039) in favour of the standard illumination protocol. Another way of assessing efficacy is by measuring the complete clearance of all AKs in a treatment area. For the standard lamp, this was achieved in 19 out of 29 treatment sides (65.5 %) as compared to 16 treatment areas with the modified illumination protocol (55.2 %). This difference was, however, not statistically significant (p=0.375).

All FU diaries were completed but did not show any statistically significant findings. However, two patients mentioned in their diaries that, even though the protocol used with the RhodoLED lamp was slightly less painful, they would prefer the Aktilite lamp in the case of needing future treatment with PDT due to the shorter duration.

4.4 Study IV

Total ion mode is performed both in in positive and negative ion mode revealing all peaks combined for different lipids, in *figure 17* positive ion mode is visualised. To make sense of all these different peaks, the data are processed in MAF to generate score pictures were there is a significant difference between the peaks. The score with the most obvious difference in distribution of lipids was MAF 1, followed by MAF 2, and so on, all the way to MAF 16 in this study. After that, the scores made no real sense biologically. However, MAF 1 is not shown because it mainly visualises differences between the tissue as a whole and the coating material of the surface.

A MAF loading score with different peaks, both positive and negative, was obtained for the studied area (picture “e” in *figure 3*, page 3 in paper IV). Five negative peaks stand out at m/z 184.07, 369.35, 542.50, 666.48 and 725.55. Negative peaks do not mean they have a negative charge, only that they stand out and form another structure (cancerous areas in this case) than the positive peaks. These peaks correspond to different lipids. In ToF-SIMS analysis of biological tissue, a commonly seen peak is at m/z 184.07, for example, and it corresponds to the phosphatidylcholine (PC) lipid head group, which is created via fragmentation of sphingomyelin (SM) lipids and PC. When the mass of an individual lipid is known, a lipid map database can be consulted to designate its nature (155). The next step is to compare the MAF score images with single ion mode images for each individual ion as seen in *figure 4*, page 4 in paper IV. Here, MAF score images are compared to the single ion images from the largest peaks in the displayed MAF scores.

Different lipids were found in different types of BCCs. Although the patients included in the study mainly had histopathologically proven highly aggressive BCCs, which warranted excision with MMS, several cases turned out to have a mixed histopathological subtype upon complete excision. For example, the specimens from patient 3 and 4 contained aggressive BCC growth as well as areas with less aggressive growing BCC. Areas with highly aggressive BCC had consistent findings of increased amounts of sphingomyelin lipids. The mixed types of BCCs, on the other hand, did not show the same increase in sphingomyelin lipids, but instead had increased quantity of PC lipids, in particular PC (32:0) and PC (34:1).

5. Discussion

5.1 Study I

The incidence of MCC has increased significantly, roughly doubling between 1993 and 2012, regardless of whether one uses the crude or different age-standardised rates. This is concordant with similar studies from other parts of the world such as The Netherlands, USA or Australia [101, 103, 104]. Studies which report on incidence rates from the 1980s often show an even higher increase during these years, but it is believed that this is, at least partly, due to unreliable detection and registration of MCC prior to the 1990s [100]. The update to SNOMED-2 in 1993 has led to better precision in the registries and the update to SNOMED-3 in 2005 even more so. This study's reported increase is therefore considered to have reliable results reflecting a true increase in the incidence of MCC during the past two decades.

Furthermore, this study supports the theory of UVR exposure as a risk factor for MCC since the majority of cases were located on sun-exposed body parts. High age is another proposed risk factor which was also supported by the findings. The lack of information on immunosuppression status in the patients who developed MCC is a weakness in the study. The SCR does not contain information regarding this. To get hold of such data, a manual assessment of all the individual patient charts would have been required. A petition to carry out such an assessment was not submitted to the Ethical Review Board since it was not likely that this would have been granted due to the potential breach in secrecy. It would also have been very cumbersome to collect all charts due to all the different electronic chart systems which exist in Sweden. Another interesting parameter to analyse would have been the MCPyV status of the MCC. However, such information is not available in the SCR and testing is not done routinely in Swedish healthcare making it impossible at the moment to study this.

Other potential weaknesses were misclassifications in the SCR or unregistered cases. These risks are present in all registry-based studies but the SCR is nationwide and reporting new cases of cancer is mandatory. In addition, the SCR has a reputation of being almost 100 % complete today. A Swedish study from 2009 found 3.7 % of the true cancer cases to be missing, i.e. the register is 96.7 % complete [156]. Moreover, two different coding systems, ICD (used by clinicians) and SNOMED (used by pathologists) were cross-referenced in order to find as many correct cases as possible.

Being diagnosed with MCC statistically implies a shorter life expectancy than one would have otherwise as presented in the results section. However, the MCC-specific death rate could not be calculated since the specific cause of death is not available in the SCR, which only contains data on death due to all causes combined. In order to calculate the disease-specific survival rate, the "Causes of Death registry" (Dödsorsaksregistret) needs to be coupled to the data set and this requires further ethical approval. This can be hard to get approval for since the limited number of MCCs per year increases the possibility of

recognizing persons in the dataset making it debatable from an ethical point of view.

When comparing the present results with neighbouring countries like Finland [102] and Denmark [100], the incidence rates in Sweden are higher and the rate of increase is more pronounced. In epidemiology studies on MCC from the USA [105, 154], the number of cases with spread disease to the lymph nodes, i.e. positive "N" status in the TNM classification, is considerably higher than in the Swedish cohort. In these studies, approximately a third of the patients (31.0-37.4 %) had positive lymph nodes at the time of diagnosis. In the Swedish cohort on the other hand, the percentage was only 9.3. This difference can most likely not be explained by differences in tumour biology but instead by the more frequent use of staging procedures in the USA [104]. Up until recently (after this study's time period), sentinel lymph node biopsy was not routinely performed in Sweden explaining why patients in Sweden have seemingly less N+ cases at the time of diagnosis. Other European studies [100, 104] demonstrate similar percentages of lymph node positive MCC patients as compared to the Swedish cohort.

5.2 Study II

PDT for BD is a relatively effective treatment with an overall clearance rate of 63.4 %. It is mostly used on body parts where other destructive treatments such as cryosurgery and C&E are less favourable due to wound healing problems or poor cosmesis (e.g. the face or the lower extremities). Also, the efficacy of all treatments also varies in BD due to, among other things, that they are user-dependent. Furthermore, comparative studies comparing different treatment alternatives for BD are limited [61]. Thus, it is not certain that destructive treatments or surgery are superior to PDT and more studies are needed to conclude this.

Risk factors for less favourable outcome in PDT for BD are only one session of treatment and larger lesions (>20 mm in diameter). Nowadays, standard treatment consists of two sessions of PDT so this is not a problem in clinical practice. Nevertheless, larger lesions should be considered when planning FU for these patients. An extra FU in larger lesions compared to smaller ones could be warranted based on the results from this study.

In other studies on PDT for BD, the reported efficacy varies from 52 to 100 % (see *Table 3* in paper II for details). Direct comparisons between studies are hard to carry out due to differences in the methodology. Some studies accept a "rescue treatment" with two more PDT sessions if the BD lesion is not healed by 3 months instead of classifying this as treatment failure. Furthermore, other studies do not count a clinical AK at the treatment site as a recurrence and, in some cases, recurrence in the area are not taken into account when reporting efficacy (i.e. the complete clearance rate is not reported).

Even though the reports on cosmetic result after PDT was not statistically significant due to too much missing data, PDT still seems to be an attractive option in this regard. In fact, 4 out of 5 patients, where cosmetic outcome had been reported in the charts, healed without any scarring or unwanted pigmentation.

This study is by far the biggest one on the subject to date which makes the data robust. In terms of limitations, the retrospective design is the greatest hurdle. Other limitations are: changes in the protocol for treating BD during the study period (e.g. the FU duration and the number of PDT sessions used), the high number of excluded patients due to incomplete charts, loss to FU or missing histopathological verification of BD and the fact that the assessment of recurrence or complete response was mainly done based on clinical findings and not histopathology. Furthermore, immunosuppression status was unfortunately not assessed in the study. Nevertheless, previous studies have not been able to demonstrate a worse outcome for these patients [157, 158].

PDT is a widely used treatment modality in Dermatology but the scientific basis for its effectiveness for BD was built on relatively small studies. This study adds more data on the effectiveness of PDT for BD and potential risk factors for predicting worse outcome.

5.3 Study III

The modified illumination protocol allowed for a small but statistically significant pain reduction during PDT for multiple AKs as compared to the standard illumination protocol, while still maintaining an efficacy over 90 %. The reported statistically significant difference in efficacy (2.5 %) between the standard and the modified protocol, in which the standard protocol came out winning, is clinically negligible. This is also demonstrated by the fact that there was no significant difference between the number of treatment sides with complete clearance indicating equivalent effectiveness with both protocols.

The relatively low amount of pain reduction with the modified protocol (1.1 VAS points) will likely not have a high impact on how PDT is performed throughout the Dermatology community. Studies on pain have shown that a 33 % reduction or a 2-point decrease on the VAS score are needed in order to be considered clinically relevant [159]. Other interventions for reducing pain in PDT, such as daylight as the light source or peripheral nerve blocks [132] have shown greater decreases in pain making the modified illumination protocol a second-hand option for selected patients.

Another factor to consider is the treatment time which is prolonged in the modified illumination protocol from 9 to almost 21 minutes. Since it is more time-consuming for nurses performing PDT, the traditional illumination protocol might be more preferable and also might allow for more PDT sessions to be performed each day. The duration of treatment can also be an important factor for the patients themselves since some patients preferred a more painful but shorter treatment.

The strength of the study is the split-face, prospective design where patients functioned as their own controls. This setup makes the pain assessment more objective. Another strength was the randomisation to which side was illuminated first and which lamp was used on this side. Furthermore, the evaluating dermatologist was blinded to the results of this randomisation at FU. The small sample size (n=29) was however a limitation. Different illumination protocols with other variations of increments in light intensity could also have been tested. This would however have required a greater number of patients, time and economic resources.

5.4 Study IV

Even though BCC is by far the most common cancer in Sweden, a lot is still unknown about its origin and biology. In the last project of the thesis, the chemical composition of lipids within a BCC was mapped for the first time. In aggressive BCCs, SMs were increased persistently while less aggressive BCCs instead showed an increase in PC lipids. ToF-SIMS provided similar discrimination between tissue types and morphological structures when compared to routine histopathological examination with H&E stainings. Deeper understanding of the biochemical profiles in different BCC types could hypothetically be of importance when developing new medications. Furthermore, finding new lipid-based markers of different skin cancers could be used for diagnostic purposes.

6. Conclusion

Based on the studies presented in this thesis, I conclude that:

- The age-standardised incidence of Merkel cell carcinoma in Sweden has increased (almost doubled) during 1993-2012.
- Photodynamic therapy is a relatively effective treatment modality in Bowen's disease.
- Modification of illumination protocols, keeping fluence rates below 50 mW/cm^2, in photodynamic therapy can lower experienced pain levels although with unclear clinical significance.
- Time-of-Flight Secondary Ion Mass Spectrometry was able to identify different lipids in healthy and cancerous tissue finding sphingomyelin lipids in aggressive basal cell carcinoma and phosphatidylcholine lipids in less aggressive basal cell carcinomas.

7. Future perspectives

Following the publication of the article on MCC, this cancer type has been discussed frequently among Swedish dermatologists, but also in the media *(figure 19)*. This has led to an increased awareness of the disease, which may contribute to an earlier diagnosis since both the patient and doctor have the clinical appearance of MCC fresh in their memory banks now more than before. My supervisor John Paoli and I have also been the initiators of a Nordic translational interest group on MCC that has now had three scientific meetings with increasing popularity. This collaboration is very important to connect researchers from different fields and enhances the awareness of MCC within the medical society. One of the planned projects within the interest group is a multicenter collaboration to study the overall survival and clinical outcome of MCC patients throughout the Nordic countries. In terms of new epidemiological studies on MCC it would be interesting to study immunosuppression and MCPyV status on Swedish MCC patients. In regards to the epidemiology of rare NMSCs, studies on incidence rates and clinical characteristics of other rare NMSCs such as atypical fibroxanthoma, dermatofibrosarcoma protuberans and Kaposi's sarcoma will be performed. The data is already collected from the SCR and the data analysis is ongoing.

Dödlig hudcancer ökar i Sverige

Publicerat måndag 23 maj 2016 kl 12.14

"Undvik direkt solljus"
(1:29 min)

Foto: Pontus Lundahl/TT

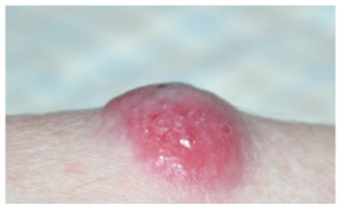
En mer aggresiv form av hudcancer ökar kraftigt i Sverige. Merkelcellskarcinom är en relativt ovanlig typ av hudcancer men enligt Oscar Zaar, hudläkare på Sahlgrenska akademin i Göteborg, är det en av de farligaste.

Läkartidningen

NYA RÖN

Kraftig ökning av Merkelcellskarcinom

Oscar Zaar, specialistläkare
John Paoli, överläkare, docent; båda hudkliniken, Sahlgrenska universitetssjukhuset, Göteborg



Egenreferat. De senaste decennierna har vi sett en närmast lavinartad ökning av antalet fall av hudcancer i Sverige. De tre viktigaste formerna av hudcancer – malignt melanom, skivepitelcancer och basalcellscancer – ökar med cirka 5–6 procent per år. Merkelcellskarcinom är en mer sällsynt men aggressiv hudcancer av neuroektodermal typ med hög återfallsfrekvens och hög dödlighet. Spridning till lymfkörtlar eller organ är

FIGURE 19. Media coverage in television, radio and several national newspapers following publication of paper I. Photo by Noora Neittaanmäki.

Regarding PDT and future aspects, our research group has just started a prospective, randomised trial comparing conventional PDT and simulated daylight PDT for AK, BD and superficial BCC. Ethical approval has been granted and patients will start being included in February 2018. Simulated daylight PDT is a new and exciting field in PDT which combines the easy and almost painless characteristics of daylight PDT with the predictability, reproducibility and possibility to treat all year round of conventional PDT. Apart from the efficacy of the new treatment modality, pain during PDT will also be assessed continuing my earlier work on pain during conventional PDT with different illumination protocols.

In terms of ToF-SIMS, new studies are also being planned. One study will continue with the already collected BCC samples but use an even higher resolution to see if this can differentiate additionally between cancer and healthy tissue. Since the method has been proven to be successful, we will also try to analyse other types of skin cancer including MM, where the impact on new diagnostic methods or drug development may be greater than for BCC. Pilot projects have been initiated to develop a routine which ensures that cancerous tissue can be collected for ToF-SIMS without risking a misdiagnosis for the patient while carrying out routine processing of biopsy specimens at the histopathology lab.

Another interesting field where my future research will be conducted is within the development of deep neural networks and machine learning based on clinical, dermoscopic and histopathological images to assist in diagnosing skin cancer. A regional research grant was just awarded for this research and ethical approval is being written at the moment.

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My other co-authors, **Bernt Lindelöf, Julia Fougelberg, Anton Hermansson, Marwa Munem, Kelly Dimovska Nilsson.**

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