ASPECTS OF FLUORESCENCE DIAGNOSTICS AND PHOTODYNAMIC THERAPY IN NON-MELANOMA SKIN CANCER

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Cover: Superficial basal cell carcinoma, fluorescence image, treatment outcome after PDT

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“*To be conscious that you are ignorant is a great step to knowledge.*”

-Benjamin Disraell, Earl of Beaconsfield, 1804-1881

This thesis is dedicated to my beloved parents, Barbro and Bror, and my loving family, Martin, Linnéa and Emma

Aspects of fluorescence diagnostics and photodynamic therapy in non-melanoma skin cancer.

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ABSTRACT

Photodynamic therapy (PDT) is now an established method to treat superficial basal cell carcinoma (BCC), Bowen's disease (BD) and actinic keratosis (AK). The main advantage of PDT is that it is non-invasive and gives excellent cosmetic results; although the majority of the patients do experience some degree of pain, which can sometimes be extreme. Fluorescence diagnostics (FD) is a method to diagnose mainly BCC, which is the most common type of tumour within the class of non-melanoma skin cancer (NMSC) and accounts for about 80% of all skin tumours. This technique can be used as an *in vivo* pre-surgical diagnostic tool, which can help to detect occult tumour borders of ill-defined BCCs.

In the first study (Paper I), the impact of fluence rate and spectral range on the primary treatment outcome and bleaching rate in AKs using aminolaevulinic acid (ALA)-PDT was studied. Pain during treatment was also registered. The results imply that the photobleaching rate and primary treatment outcome were dependent on the fluence rate and that a low fluence rate (30 mW/cm^2) appears preferable. In the second study (Paper II), risk factors related to pain during PDT for AK were investigated. The most important factors relating to the experience of pain seem to be the size and "redness" of the lesion. No significant pain relief with capsaicin was seen. In the third study (Paper III), the transdermal penetration of ALA and methyl-aminolaevulinate (MAL) *in vivo* were investigated using a microdialysis technique. The results imply that there is no significant difference in transdermal penetration of ALA and MAL in tumour tissue. Detectable levels of the drug were not obtained in almost 50% of the lesions where catheters were inserted 1-1.9 mm into the lesion. Curettage was not found to affect the interstitial concentration, indicating that penetration of the drug might indeed be a problem when treating BCCs thicker than 1 mm. In the final study presented within this thesis (Paper IV), the fluorescence contrast in patients undergoing MAL-PDT for superficial BCCs was evaluated. The MAL fluorescence contrast obtained between the tumour and normal skin was also compared to that obtained in a previous study using ALA. In both cases it was possible to identify areas in the fluorescence images corresponding to a tumour and to surrounding normal skin. The mean fluorescence contrast with MAL, however, was significantly higher than the mean fluorescence contrast after application of ALA. Thus, MAL generally renders a higher tumour contrast compared to ALA in superficial BCCs. No correlation between fluorescence and treatment response could be observed.

The results of this thesis prove that PDT, using either ALA or MAL, is effective in the treatment of thin non-melanoma skin cancer and pre-cancer. These results further suggest that lower fluence rate should be considered as a precaution to minimise pain response when treating large and inflammatory lesions, although more study is needed. When performing FD, MAL is the best option and lack of treatment response cannot be connected to fluorescence but maybe due to the fact that the pro-drug does not successfully penetrate into the deeper parts of the tumour.

***Key words*:** actinic keratosis, aminolaevulinic acid, fluorescence contrast, methyl-aminolaevulinic acid, microdialysis, non-melanoma skin cancer, pain, photodynamic therapy

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LIST OF PAPERS

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

I. Ericson MB, Sandberg C, Stenquist B, Gudmundson F, Karlsson M, Ros A-M, Rosén A, Larkö O, Wennberg A-M and Rosdahl I. Photodynamic Therapy of Actinic Keratosis at Varying Fluence Rates: Assessment of Photobleaching, Pain and Primary Clinical Outcome. *Br. J. Dermatol.*, 2004; **151:**1204-12.

II. Sandberg C, Stenquist B, Rosdahl I, Ros A-M, Synnerstad I, Karlsson M, Gudmundson F, Ericson MB, Larkö O and Wennberg A-M. Important factors for pain during photodynamic therapy for actinic keratosis. *Acta Derm. Venereol.*, 2006; **86**:404-8.

III. Sandberg C, Halldin C, Ericson MB, Larkö O, Krogstad AL, Wennberg A-M. Bioavailability of aminolaevulinic acid and methyl-aminolaevulinate in basal cell carcinomas - a perfusion study using microdialysis *in vivo. Br. J. Dermatol.*, 2008 Nov **159(5)**:1170-1176, E-pub 2008, Aug 19.

IV. Sandberg C, Paoli J, Gillstedt M, Halldin CB, Larkö O, Wennberg A-M and Ericson MB. Fluorescence diagnostics in connection to photodynamic therapy with a comparison of methyl-aminolevulinate and aminolevulinic acid. Submitted for publication

CONTENTS

ABBREVIATIONS

5-FU, 5-fluorouracil
ALA, aminolaevulinic acid
AK, actinic keratosis
BCC, basal cell carcinoma
BD, Bowen's disease
CCD, charged coupled device
D, dose
FC, ferrochelatase
FD, fluorescence diagnostics
HPD, hematoporphyrin derivative
HPLC, high-pressure liquid chromatography
I, intensity
J, joule
KA, keratoacanthoma
LED, light emitting diode
MAL, methylaminolaevulinate
MD, microdialysis
MSC, melanoma skin cancer
mW, milliWatt
NBCCS, nevoid basal cell carcinoma syndrome
NMSC, non-melanoma skin cancer
PBGD, porphobilinogendeaminase
PpIX, protoporphyrin IX
PDT, photodynamic therapy
ROI, region of interest
ROS, reactive oxygen species
RR, relative recovery
SCC, squamous cell carcinoma
Succinyl-CoA, succinyl-Co-enzyme A
T, time
UV, ultraviolet
VAS, visual analogue scale

1. INTRODUCTION

1.1. Human skin

The skin is the largest organ of the human body, see **figure 1.** It protects the body from water loss, regulates body temperature and is the outer barrier against heat, cold, and bacteria. Stretched out it has an area of approximately 2 m^2. The skin is divided into three main layers: the epidermis, the dermis and the subcutis[1]. This thesis will deal mostly with the two upper layers of the skin as this is where photodynamic therapy (PDT) and fluorescence diagnostics (FD) take place.

The epidermis, is the thin top layer approximately 0.1 mm thick[1]. The deepest part is the *stratum basale*, containing immature cubic cells of the keratinocytes. As these cells divide and mature, they move to more superficial layers, first to the *stratum spinosum* and then to the *stratum granulosum* where these cells become more extended. Finally, the keratinocytes become hornified in the most superficial layer, *stratum corneum*, where they lose their nuclei. This horny layer forms the "semi-permeable membrane" of the body and protects it from water loss [2]. In the *stratum basale* there are also dendritic cells which are placed at an interval of every 10th cell, so called *melanocytes* [3], forming the melanosomes which contain pigment. It is these melanosomes that are transported out to the surrounding keratinocytes and protect them from UV-radiation. Each melanocyte will support 36 keratinocytes with melanosomes [4]. Another dendritic cell is the Langerhans cell. This cell is an antigen presenting cell which processes exogenous antigens which are then presented to naïve T-cells. The Langerhans cells account for approximately 3-6% of the epidermal cells[1].

The dermo-epidermal junction separates the epidermis from the dermis. The papillary dermis is the upper part of the dermis where capillaries extend from the superficial plexus of blood vessels, dividing the papillary dermis part from the reticular dermis.

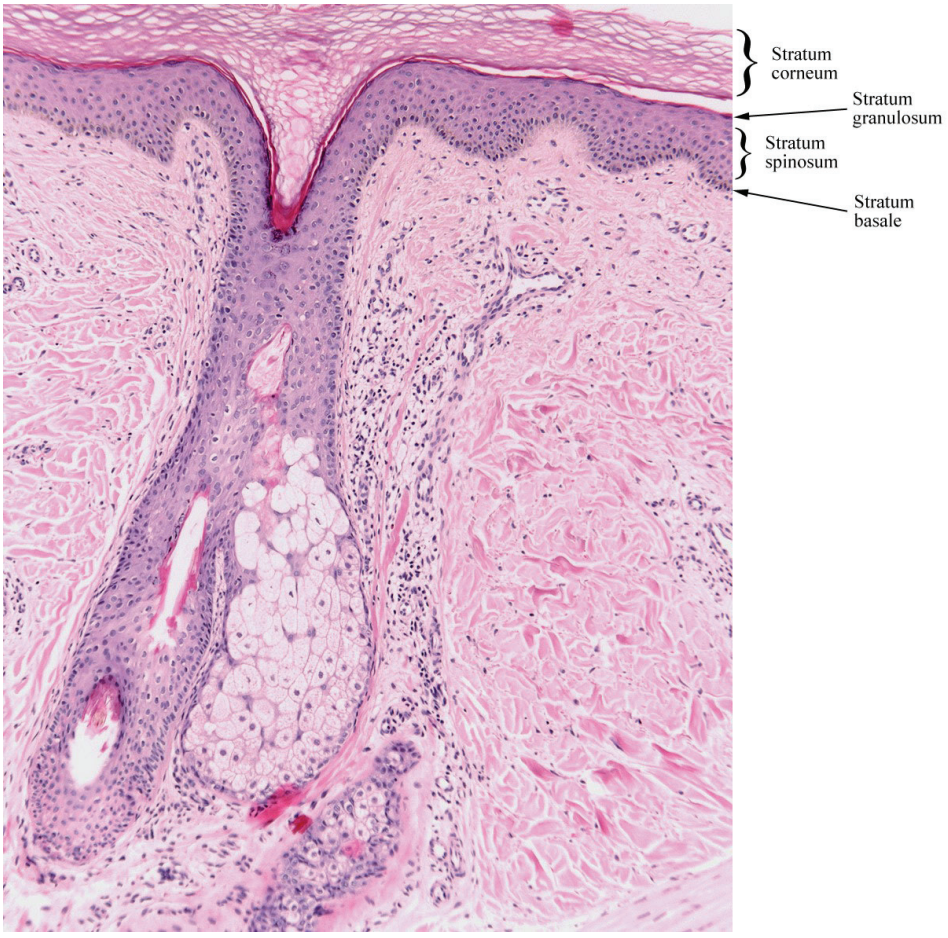

Figure 1. Skin anatomy **(**Photo : Lena Mölne)

The dermis is composed mostly of fibres produced from fibroblasts. These fibres (elastin, collagen, reticulin) are surrounded by a liquid ground substance consisting of water, glucosaminoglycans and hyaluronic acid. Together these substances provide the elasticity and

strength of the skin. In the dermis, the blood vessels also support the epidermis and the dermis with nutrients and oxygen.

The subcutis, which is the deepest part of the skin, mainly consists of lipocytes and some blood vessels. The subcutis functions as an energy reservoir and thermo-insulator of the body. To some extent it also protects the body from trauma.

1.2. Skin cancer

There are two main groups of skin cancers: melanoma skin cancer (MSC), and non-melanoma skin cancer (NMSC); although some other rare forms of cancer do exist such as dermatofibrosarcoma protuberans, Merckel cell carcinoma and atypical fibroxanthoma. This thesis, however, will focus on the two common types of cancer, i.e., MSC and NMSC.

1.2.1 Melanoma skin cancer

MSC originates from pigmented cells, the so called melanocytes. The pigment, melanin, absorbs light mainly in the visible spectrum (400-600nm), the same spectrum of light that is used in both PDT and FD. Therefore only red light (around 635nm) is theoretically suitable for PDT of melanoma cells. This treatment is not recommended for melanoma cells, however, as while *in vitro* melanoma cells have responded to the treatment, *in vivo* melanoma cells respond very poorly[5,6]. The FD-method is not applied to pigmental skin cancer as the absorption spectrum for FD is UV-blue-light, i.e., the same spectral region that melanin absorbs light.

1.2.2 Precursors to non-melanoma skin cancer

1.2.2.1 *Actinic keratosis*

Actinic keratosis (AK) is the earliest precursor for squamous cell carcinomas, SCCs, although few AKs actually proceed to SCCs. Approximately 25% of AKs regress spontaneously, while about 10% will become SCCs [7]. The AK consists of disordered keratinocytes with cytological atypia, parakeratosis and loss of the *stratum granulosum*. Clinically, AKs can be classified in three grades: I) those not easily seen but with a rough feeling of the skin, II) those easily seen as a red scaly area with a rough skin and finally III) obvious, hyperkeratotic, indurated red scaly areas [8]. Often, the AKs derive from sun-exposed and sun-damaged skin [9]. The AK's can, therefore, appear over an extensive area which is often described as field cancerization, i.e., a field full of potential SCCs.

1.2.2.2 *Squamous cell carcinoma in situ*

Squamous cell carcinoma *in situ*, i.e., Bowen's disease, is the final precursor for squamous cell carcinoma. Histopathologically, the atypical keratinocytes constitute the whole of the epidermis but have not yet broken the basal membrane. After the breakage of the basal membrane they have formally become a true SCC. Bowen's disease often arises from actinic keratosis. Clinically it can imitate eczema and appear as a red scaly well-defined area. Usually it is located on sun-exposed areas such as the upper extremities, ears and cheeks [10,11]. SCC has a clear relationship to sun-exposure, i.e., UV-B radiation. Other risk factors include immunosuppressive medication, HPV, chronic scarring and inflammation of the skin[11-13]. All non-melanoma skin cancer and NMSC-precursors are more common in males than females[9].

1.2.3 Non-melanoma skin cancer

1.2.3.1 *Basal cell carcinoma*

Basal cell carcinoma (BCC) represents approximately 80% of all non-melanoma skin cancers. BCCs are not thought to have any precursors, i.e., they arise *de novo*. Risk factors for BCCs include cumulative and intense "bursts" of exposure to UV-light, hereditary factors including skin type, exposure to arsenic, radiation and immunosuppressive medication. Most BCCs (85%) appear on the head and neck and (fortunately) very rarely metastasize. Usually growth is slow. For all BCCs, the mean age of occurrence is between 60-70 years of age[14].

There are three main forms of BCCs, defined according to their histological growth: nodular, superficial and infiltrative including morpheiform.

Nodular BCCs account for approximately 50-60% of all BCCs. These are more common in males and occur mostly on the head and neck. Clinically, they appear as pinkish nodular shiny tumours, often with telangiectasia. Histopathologically, the tumour nests extends into the dermis.

Superficial BCCs account for approximately 25% and are more common in younger females. Superficial BCCs tend to be more equally distributed on the body, often on the trunk in men and upper extremities in females. They appear as maculae and have a pinkish colour with associated scaling. Tumour nests grow in connection to the basal cell layer and the hair follicles.

Infiltrative including morpheiform BCCs account for approximately 15 % of all BCCs. These are mostly situated in the head and neck region and are the most aggressive form of BCC

which grows in an infiltrative manner. Clinically morpheiform BCCs can look like a scar with diffuse extension and ill-defined borders. This is one of the reasons why they are often not discovered until very late when they may have extended into the subcutis, muscle tissue or bone.

1.2.3.2 Basal cell nevus syndrome (Gorlin syndrome)

Nevoid basal cell carcinoma syndrome (NBCCS) is caused by mutations in the PTCH1 gene. The PTCH1 gene encodes the principle receptor for the Hedgehog signalling pathway. NBCCS is equally common in males and females. Mean age of onset is 25 years old but the syndrome can occur early in childhood. The main clinical manifestations (major criteria) include: multiple basal cell carcinomas (BCCs), odontogenic keratocysts of the jaws, hyperkeratosis of palms and soles seen as palmar and plantar pits, skeletal abnormalities, first degree relatives with NBCCS. Minor criteria include: facial dysmorphism, radiological abnormalities, medulloblastoma and ovarian fibroma. Diagnosis is set if two major criteria are fulfilled or one major criterion and two minor criteria. The BCCs are most commonly located on the face, back and chest and are more frequent on sun-exposed areas[15].

1.2.3.3 *Squamous cell carcinoma*

Squamous cell carcinoma (SCC) is a tumour that derives from the keratinocytes in the epidermis. Clinically they appear in actinic skin in elderly people growing to fully developed skin cancer invading the dermis. SCCs often appear to be a plaque growing to form a nodule and finally ulceration. The main distribution is on sun-exposed areas such as the head, neck and upper extremities including the dorsum of the hands. SCCs have a clear relationship to sun-exposure, i.e., UV-B radiation. Other risk factors include: immunosuppressive medication, HPV, chronic scarring and inflammation of the skin [12,13]. SCCs are more common

in males than females[9]. In a large study, Brantsch *et al.* (2008)[11] found the mean age of onset to be approximately 70 years of age

Generally SCCs are slow-growing with a potential to metastasize, especially when located on the ears and lips. SCCs can be well, moderately or poorly differentiated; the last of the three types having the worst prognosis.

1.2.3.4 Keratoacanthoma

Keratoacanthoma (KA) is a tumour with a peak incidence between 50-69 years in which both genders are equally affected. Ethiology is unknown but one theory is that these tumours are derived from hair follicles because of the large amount of keratin stored there. Clinically, they appear mostly on sun-exposed areas such as on the face, neck and hands. Risk factors include a light (pale) complexion, UV-radiation, trauma, chemical carcinogens and immuno-suppressive medications[16,17].

These tumours grow rapidly up to 25 mm in size in 6-8 weeks and start as a small pink macule, which forms a nodule with a keratin-filled volcano-like crater. During involution, approximately 50% of all tumours disappear. Some lesions may exist for years before disappearing. Typically the whole cycle takes 4-9 months. An atypical KA can, however, be very aggressive. Microscopically it is very difficult to differentiate between a KA and an SCC. KA is therefore treated as an SCC[18].

1.3 Treatment of non-melanoma skin cancer and its precursors

National guidelines refer to the treatment of all precursors and NMSC[19-22].

There is an ongoing debate about the importance of treating all actinic keratoses. However considering the above facts most authors recommend treatment. The treatment of choice is often dependent on the size of the lesion. Large areas of AKs are very suitable for PDT, but even topical therapy, such as imiquimod and 5-FU, is effective[23]. Sometimes diclofenac [24]can also be used, but this seems to be less effective. However, PDT is typically a "one-shot therapy", and is therefore safer than topical treatment. Further, several studies have shown compliance with recommended drugs is low which compromises their function[25,26]. Cryosurgery with liquid nitrogen is the preferred treatment of choice for *single* AK lesions; but is not recommended as treatment for field cancerization.

Squamous cell carcinoma in situ is a superficial lesion suitable for skin excision, cryosurgery or electro-dessiccation with curettage, PDT or 5-FU. Choice of treatment depends on location, size, extent of the lesion and local guidelines.

Basal cell carcinomas are treated according to their histological growth pattern (nodular, superficial and infiltrative (morpheiform)) and local guidelines.

Nodular BCCs can either be excised, or treated with cryosurgery or electro-dessiccation preceded by curettage. PDT can be considered for thin nodular BCCs.

For *superficial BCCs***,** PDT and imiquimod are excellent therapies due to the good cosmetic outcome. Cryosurgery is also an option, and sometimes excision is used.

Finally for *morpheiform BCCs* with infiltrative growth, Mohs micrographic surgery is recommended. However, excision with clear margins according to local guidelines can also be performed[27].

Nevoid basal cell carcinoma syndrome (Gorlin syndrome)

The treatment of BCCs is based on the histopathological growth. Surgery is indicated when the number of lesions is limited or with aggressive growth. As the number of BCCs increases, individual schemes may be needed and other treatments including laser ablation, photodynamic therapy and topical chemotherapy, are often used. However, radiotherapy should be avoided. Not only are recurrences and incomplete clearance rates high when using radiotherapy but the PTCH1 gene is also susceptible to the radiation and may be prompted to produce more BCCs. Even in healthy patients, induction of BCCs in skin treated with ionizated radiation have been reported[15,28].

Squamous cell carcinoma and *keratoacanthoma* should always be excised with margins based on histologic investigation and local guidelines.

Radiotherapy is an option in the treatment of NMSC but is seldom used due to the disadvantages with scarring and the high rate of recurrence. Recurrences occurring after radiotherapy of BCCs often require Mohs surgery[27,29].

1.4. Photodynamic therapy (PDT)

1.4.1 History and basic aspects of PDT

The history of PDT is more than a hundred years old. In the 1890s a Danish scientist, Niels Finsen, treated skin tuberculosis with sunlight. The treatment was successful and for this discovery he received the Nobel Prize in 1903. The success of Niels Finsen created scientific interest worldwide and in the beginning of the 20th century an intensive research period, involving the use of sunlight, began. In 1903 two scientists, Hermann von Tappeiner and Albert Jesionek, treated BCCs with the dye eosin and afterwards irradiated with visible light. This treatment also proved to be successful [30] .

In the 1970s Thomas Dougherty duplicated the work of von Tappeiner and Jesionek but used hematoporphyrin derivative (HPD) in the treatment of skin cancer. HPD was administered systemically, the reason for this being that the HPD molecules were too large to be able to penetrate the skin topically[31]. The major disadvantage of this treatment was the photosensitization of the skin for weeks following initial treatment[31]. In 1990 Kennedy and Poitiers described what would become today's modern topical PDT using a porphyrin-precursor, aminolaevulinic acid, ALA, as a sensitizer for superficial BCCs and then irradiating the effected area with visible light[32].

The basic principles of PDT are the administration of a pro-drug or a photosensitizer such as ALA, either topically or intravenously, to rapidly dividing cells such as, e.g., atypical cells and tumour cells. When sufficient sensitizer has accumulated in the target cells, the effected

area is irradiated with a regulated dose of light, with an appropriate wavelength to match the photosensitiser's peaks of absorption. An energy transfer between the sensitiser and molecular oxygen will take place which will form reactive oxygen species (ROS), mainly singlet oxygen. The cells are then destroyed. However, the exact mechanism for the destruction of the cells is not yet fully understood, although the mitochondrial membrane is most likely disrupted when using ALA-PDT. Since both PDT and FD are relatively new techniques they still need to be improved and further investigations are needed to fully understand the mechanism of action.

1.4.2 Photosensitization

1.4.2.1 *Photosensitization using ALA and MAL*

The δ-5-aminolaevulinic acid (ALA) or its methyl-ester, methyl-aminolaevulinate (MAL) are most commonly used for PDT in dermatology. Other sensitizers from the chlorine-, phtalocyanine- and phenothiazinium- families may also be used. These will not be further discussed in this thesis.

By using the haem biosynthesis in the cells the final photosensitizer, protoporphyrin IX, PpIX, is endogenously formed, see **figure 2.** As seen in **figure 2**, the metabolization is localised to the mitochondria and the cytosol. In the haem biosynthesis ALA is syntesized by succinyl-CoA and glycine in the mithochondria, which is regulated by a negative feedback from the last step where iron is incorporated to build haem. By adding ALA exogenously, the first step and regulation are bypassed and more PpIX can be formed.

The PpIX is believed to accumulate more rapidly in the tumour cells than in the normal cells due to an altered skin barrier in the tumour cells. Further, differences in enzymes between tumour cells and normal cells cause more rapid accumulation of PpIX. Finally, tumours may

have increased blood perfusion fostering the accumulation of PpIX. In tumour cells the skin barrier is disrupted [33] and is thought to have a more rapidly functioning porphobilinogen deaminase (PBGD) and a less rapidly functioning ferrochelatase (FC) than normal cells [34-36]. Therefore when enough PpIX is formed in the tumour cells, the normal cells have still not accumulated enough photosensitizer to be damaged by the photochemical reaction.

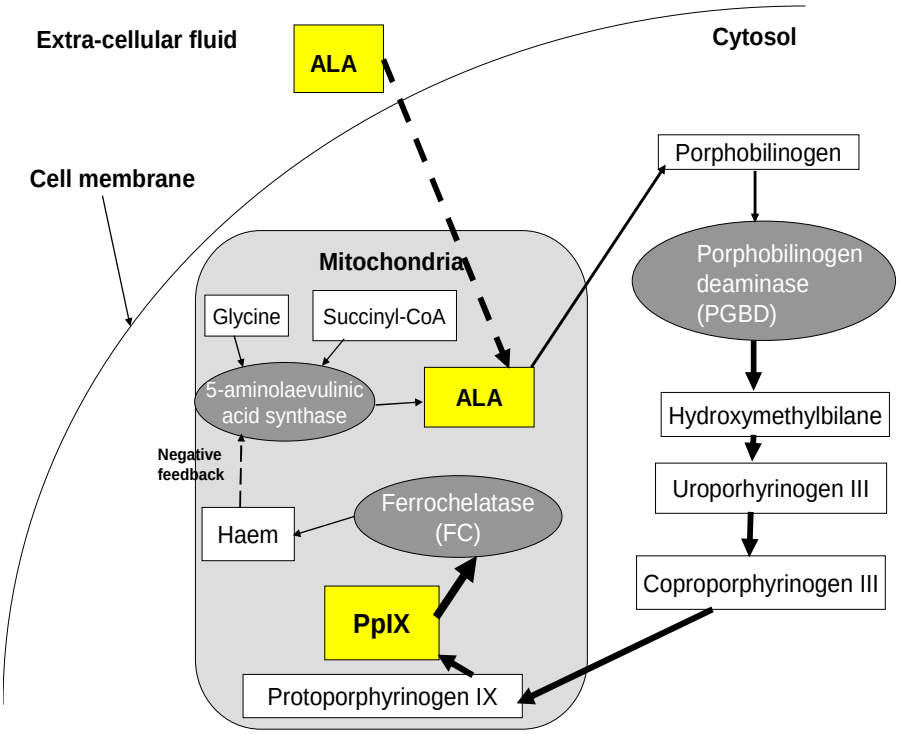

Figure 2. Haem biosynthesis

1.4.2.2 Penetration of the pro-drug

One of the disadvantages with topical PDT is that the administration of the pro-drug can be difficult. ALA is a hydrophilic and charged salt-substance. Therefore, penetration deep into the skin can be difficult since the *stratum corneum* consists of cell remnants, with hydrophobic cell membranes and will not easily permit the ALA to diffuse passively. Once the *stratum corneum* is penetrated, however, ALA can travel two pathways, either between the cells in the extra-cellular fluid as the extra-cellular fluid is more hydrophilic, or through active transport of ALA into the cells via the GABA-receptors[37]. MAL is more hydrophobic and will hypothetically more easily penetrate the *stratum corneum* and through the cell membrane. The *stratum corneum* is very hydrophobic, therefore in theory there is a greater risk that MAL will be trapped there and therefore not be able to penetrate deeper than ALA. Furthermore in contrast to ALA, MAL is not actively transported into the cells via the GABA-receptors[37] therefore ALA but not MAL can be transported directly into the nerve-end fibres and make the skin area more sensitised. This may be one reason why greater pain is experienced during ALA-PDT (see the section 1.4.7).

As there is a limit to how far both ALA and its esters can reach into the skin there is some uncertainty whether they can reach deep enough to treat the whole tumour and therefore only superficial lesions are suitable candidates for PDT. This is thought to be one of the explanations for the limited cure rate of nodular BCCs using ALA-PDT[38,39]

There are methods for recording the depth that the pro-drug and PpIX have reached. Almost all of these methods have been developed in *in vitro* studies. The pro-drug is applied topically on the normal skin or on tumour tissue. After a specified period of time a biopsy is taken and examined in a fluorescence microscope. Peng *et al.* [40] detected PpIX, when examining six

(human) biopsies, down to a depth of 2 mm in only one of the biopsies. Ahmadi *et al.* [41] in an *in vitro* study using scintillation spectroscopy and a precise tissue sectioning protocol found that out of five human BCC-biopsies only two showed ALA at 2 mm depth. Until now there has been only one *in vivo* study, by Wennberg *et al.*[42], where ALA was detected in superficial BCCs by microdialysis at a depth of 0.5 mm.

1.4.2.3 Pre-treatment of the lesion

To overcome the difficulties of penetration, a light curettage of the lesion is widely performed without any bloodshed. The rationale is to remove the *stratum corneum* and hyperkeratoses to ensure easier penetration although different studies have shown varying results. Other techniques such as tape-stripping may also be used. Van den Akker [43] described in a study in 2003 that tape-stripping of normal nude mouse skin increased the PpIX-concentration implying that *stratum corneum* is an important barrier for the pro-drugs. This was supported by a study from Gerritsen[44] and in a study by Smits *et al.* [45] who showed a negative correlation between the thickness of *stratum corneum* and a higher amount of superficial PpIX measured indirectly by fluorescence diagnostics. As for superficial BCCs and BDs it does not seem necessary to pretreat. Moseley *et al.* [46] found in 2008 that there was no significant difference in superficial PpIX fluorescence in superficial BCC and BD with or without prior curettage of the lesion nor were there any significant differences in the cure rate. Still the use of curettage before applying the pro-drug is widely performed and further studies on this subject are required to confirm the earlier results.

1.4.2.4 Treatment time with ALA and MAL

Accumulation of PpIX is a time-dependant reaction [47,48]. Many investigations have been made to determine how much time is required to ensure sufficient accumulation of porphyrin to perform PDT. Some authors have used fluorescence investigations to find out when the highest contrast value is reached, i.e., when the highest concentration of PpIX has been achieved. Ericson *et al.* (2003)[49] investigated BCCs using ALA and studied fluorescence contrast and threshold limits 1-4 hours after application time of ALA. It was shown that the highest contrast value between the BCCs and normal tissue was achieved with 3 hours pre-treatment with ALA. In Europe this application time has become standard both with ALA and MAL. Later studies have questioned whether pre-treatment for 3 hours is really necessary. Braathen *et al.* [50]performed a PDT-study of the AK cure rate, comparing 1.5 h application with 3 h application. They found that, although 1.5 h of application had slightly lower cure rate than 3 h, the cure rate was still sufficient. Sakamoto (2009)[51] found a progressive increase of the contrast value for PpIX between 30 to 120 min after the application in an *in vitro* investigation on swine skin after topical application of ALA. Despite these publications further studies are needed before a shorter application time than the typical 3 hours could be recommended.

1.4.2.5 Enhancing the penetration

Penetration enhancement technology is presently developmental. The permeation of drugs through the skin can be enhanced either physically with iontophoresis (i.e., the application of low-level electric current), occlusion, excess of substance, tape-stripping/curettage; or chemically using permeation enhancers (CPE) with fatty acids, alcohols or terpenes which change the physicochemical structure of *stratum corneum*. Furthermore, for specific substances the interference may involve certain processes such as the iron chelators which

interfere with the haem biosynthesis in ALA-PDT[44,52] and may increase the production of PpIX. A high skin temperature, which increases the permeation of ALA, may also speed up the PpIX-production and the pH-level also influences the PpIX-production. However, none of the above mentioned measures are as yet in clinical practice[53].

The most common enhancer used clinically, with the exception of the removal of *stratum corneum*, is a 3 hour occlusion of the treated area an excess of the pro-drug, covered with a thin plastic film.

1.4.3 Photophysics

PDT can be divided into three main processes: sensitization, illumination and production of reactive oxygen species (ROS). All these processes are necessary to in the end finally cause tissue-death. The main treatment outcome of PDT is dependant on how much ROS such as singlet oxygen are produced in the cells[54]. To produce ROS, molecular oxygen and energy are required. Chromophores, like porphyrines, are able to absorb energy when irradiated by light [55]. Different chromophores require different wavelengths according to their absorption spectra. As all molecules strive to resume the ground state, energy is dissipated mainly by vibrational relaxation see **figure 4**, page 28. But to some extent fluorescence is obtained, i.e. the emission of longer wavelengths. For certain chormophores, such as porphyrins, energy can be transferred to surrounding molecular oxygen when trying to resume its ground state, resulting in the production of singlet oxygen.

The absorption curve for PpIX has five peaks. The main peak, known as the Soret-band, absorbs the most energy at approximately 400 nm. There are 4 more peaks throughout the rest of the visible spectrum, these peaks are called the Q-bands, see **figure 3**.

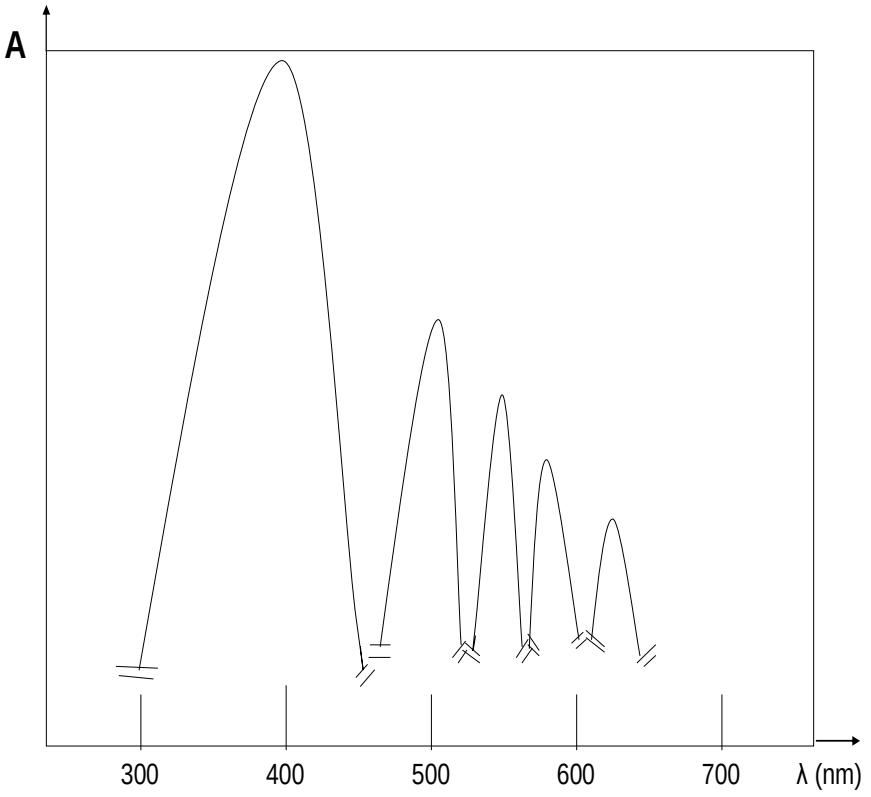

Figure 3. PpIX Absorption curve (not in scale)

The longer the wavelength, the less energy is absorbed. When the PpIX has absorbed energy from the light it becomes excited from the ground state see **figure 4**. The PpIX quickly releases this energy in order to resume the ground state. . The PpIX fluoresces in the red part of the spectrum. However, for some molecules, energy from the donor PpIX is transferred to molecular oxygen forming singlet oxygen. The singlet oxygen will react with surrounding molecules. This causing the desired oxidative stress in PDT, ultimately destroying the tissue.

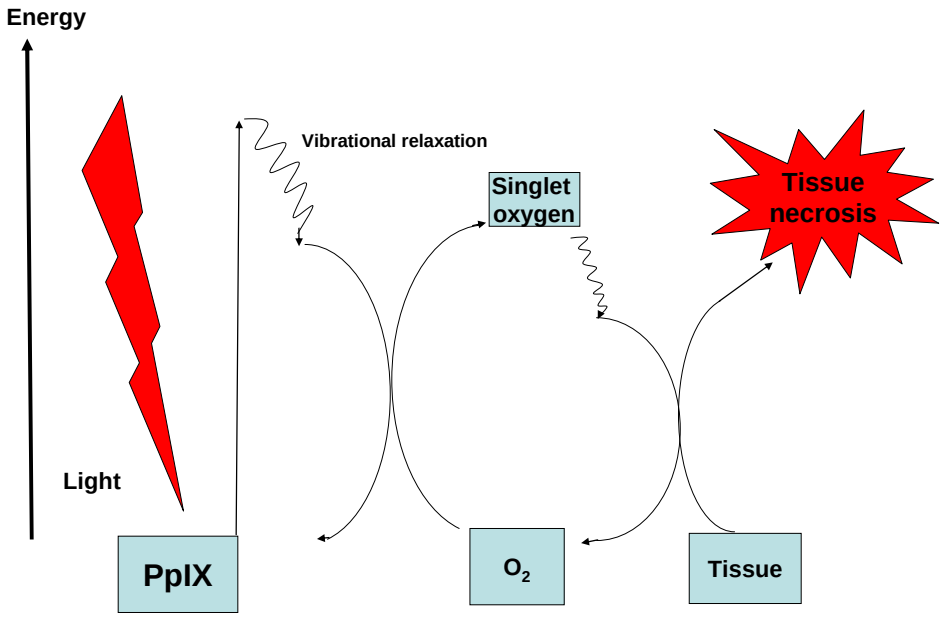

Figure 4. Energy transfer at PDT

1.4.4 Photobleaching

When light irradiates the photosensitizer PpIX, the formed singlet oxygen can react with the sensitizer itself in a photochemical reaction called photobleaching. In this reaction, the photosensitizer is consumed. Photobleaching is an oxygen-dependent process [56]. This means that when the oxygen is depleated no photobleaching can take place. To monitor the photobleaching *in vivo,* fluorescence diagnostics can be performed as will be further discussed in section 1.5. Fluorescence images can be taken and image analyses can be used to calculate how much PpIX remains. Langmack *et al.* [57] proposed the idea that the rate of photobleaching is dependent on the fluence rate.

1.4.5 Dosimetry

A specific dose (J/cm^2, energy per unit area) is irradiated on the target, the dose is dependent on the fluence rate, i.e., if the Intensity = I (mW/cm^2) and the treatment time Δt then one can express the dose dependence as:

$$D = I \Delta t$$

The accumulated dose depends on which light source is used. Different light sources emit light over different spectral regions. When comparing two light sources with different spectral characteristics the total light dose will not be comparable, i.e., the dose for different light sources will depend on the phototherapeutic efficiency of the given spectral range they emit. The phototherapeutic efficiency of a light source can be defined as the number of excitations per second of the photosensitizer [58]. It is therefore not easy to compare the dose between the different light sources without a thorough characterisation of the excitation efficiency of each source. There are different efficiencies for different light sources. The effective fluence light is defined as the light energy which is absorbed by the sensitizer. It should be noted, however, that some results have been described in the literature which show that high fluence rate is not to be recommended due to hyperthermia which seems to depleat oxygen more rapidly[57,59]. Light fractionation has been tested with positive results. Star (2006)[60] saw that a two-fold PDT-illumination with a 2 hour dark interval between the two sessions gave a very high 2 year complete remission. When de Haas (2008)[61] investigated the fluorescence microscopically with fractionated light he was unable to fully explain the increased amount of PpIX between the two treatments. However Puizina-Ivics (2008) [62] proposed that fractionated illumination may improve cure rates due to re-oxygenation between the fractionated treatments.

1.4.6 Light sources for PDT

There are a wide range of different light sources that can be used for PDT. Both non-coherent light, e.g., example metallo-halogenes and light emitting diodes (LED), and coherent light, e.g., lasers, have been used. In recent years sunlight has also been investigated with good results [63,64]. In some countries, for instance in the USA, blue light (405 nm) is used; while in Europe red light (635 nm) is favoured. The blue light has higher absorption by PpIX compared to red light; however the penetration of red light is better. The penetration depth (defined as the depth where the intensity has decreased to a factor 1/e) is only approximately 0.1 mm for blue light into the epidermal tissue[65,66], compared to values ranging from approximately 0.55 mm to 1/e [66] up to 5 mm[65] for red light.

Every light source has its advantages and disadvantages. Broadband and filtered non-coherent light sources have the advantage that they can be used to treat a large area at the same time, that they are easy to handle and inexpensive. The disadvantage of this treatment is the wide range of wavelengths.

Monochromatic coherent light sources, such as lasers, have the advantage of using an exact wavelength. This is the preferred light source for fibre-optics, when internal organs are the target. The disadvantages are that they are expensive and only a small area can be treated at once.

LED is a more recent light source which can be used both as diode lasers but also like non-coherent light, the advantages being a narrower wavelength-band than metallo-halogenes. In

recent years an ambulatory low-irradiance organic LED light source has shown very successful results [67].

1.4.7 PDT and pain

Generally there are few side effects with PDT, the major side effect being pain. The treatment can be experienced as a very unusual burning or stinging sensation. Clinically the onset starts early during illumination and as treatment stops the pain decreases dramatically, but, in some cases it can last for 12-24 hours or more after conclusion of the treatment. The pain is usually measured by a VAS-scale ranging from 0 (no pain) to 10 (worst pain imagined).

Grapengiesser *et al.* (2002)[68] found that the level of pain is normally distributed among patients and risk factors for pain include: 1) size of the area treated, i.e., the larger the area treated the greater pain experienced, 2) diagnosis, i.e., AKs are often more painful to treat than BCCs and BDs, and 3) location of lesion, i.e., scalp, genital area and lips. Wiegell *et al.*[69] investigated fluorescence, which was found to be correlated to pain (the greater the accumulation of PpIX, the more pain). In the same study she also showed that lower fluence rate resulted in a lower experienced pain. Red light has been shown to be more painful to the patient than green light [70], "high-output" broadband seems to be more painful than "low-output" broadband [71], and finally fractionated light treatment has been demonstrated to be less painful [72] than non-fractionated.

Over the years many attempts to reduce pain have been tried unsuccessfully including the application of topical local anaesthetics like morphine-gel [73], tetracaine-gel [74] and lidocaine-prilocaingel [75] [68].

Some papers have suggested that ALA is more painful than MAL[76-78]. The reason for this has been suggested to be due to the fact that ALA has an active transport into the cells which MAL does not have. Water, cold-packs, cold air analgesia (-35°C) and transcutaneous electrical nerve stimulation, TENS, have been administered with modest success [72,79,80] to alleviate the experience of pain in conjunction with the treatment.

Recently successful treatment against pain has been found. Sub-cutaneous infiltration-anaesthesia [81], nerve blocks [82,83], sun as a light source[63] and ambulatory PDT using organic LED light source [67] have all shown good pain relief. Some of these treatments have disadvantages such as heavy oedema and the need for injections. Sunlight requires good weather, which makes planning difficult in countries which do not have a high frequency of sunlight. So far nerve blocks provide the most efficient pain relief during PDT for lesions on the scalp, face and genitals and the least disadvantages. Finally, good nursing and a friendly atmosphere during treatment have been shown to make PDT easier to tolerate.

1.5. **Fluorescence diagnostics (FD)**

1.5.1 Basic principles

Fluorescence diagnostics is a non-invasive *in vivo* method used to selectively differentiate neoplastic tissue from healthy skin [84,85]. As with PDT, FD uses the fact that tumour tissue has different properties compared to normal tissue; i.e., a destroyed outer barrier, more rapidly dividing cells, altered enzymes and possibly increased blood perfusion[47]. All these properties together enable tumour tissue to be distinguished from healthy tissue. The principles include excitation of fluorophores with light of a certain wavelength and afterwards the detection of

the fluorescence, either by endogenous fluorophores, autofluorescence, or by induced fluorescence, e.g., ALA induced fluorescence.

The fluorescence diagnostics that we know it today has been developed lasting recent decades. However it was noted as early as 1924 when Policard reported red fluorescence obtained from a tumour on a rat by irradiation with Wood's light [86 87] . After this finding, research was intensified. Spectroscopy was studied and sensitive spectrophotometers were developed, and using these, point measurements could be made[88]. With the development of charged coupled devices (CCD) connected to a computer it became possible to visualise the tumour using image analysis [89,90] see **figure 5**.

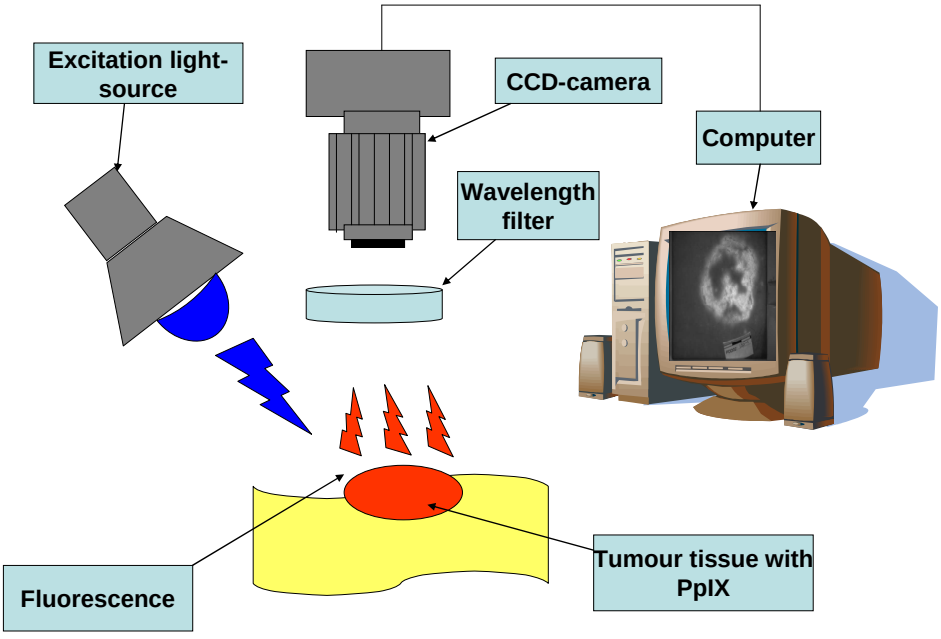

Figure 5. Fluorescence diagnostics

Light sources to use in conjunction with ALA and MAL need to emit light in the UV-blue light range since their maximum absorption is at 405 nm. Both coherent, laser and non-coherent light can be used, as described previously for PDT in section 1.4.6. Which light source is used depends on the location of the organ to be treated; e.g., in hollow organs only lasers can be as these are suitable for use in optical fibres. For skin tumours both lasers and non-coherent light such as metallo-halogenes and LEDs may be used as some dispersion is not only allowed but may even be advantageous. As for PDT, the same advantages and disadvantages must be considered.

Depending on the desired wavelength, a CCD-camera with different filters is most commonly used. Using this tool the intensity can be detected over a 2-dimensional area. The CCD-camera is connected to a computer to make image analysis possible. The data consists of grey-scale images where each pixel value corresponds to the measured intensity value. This can be converted to a colour-scale showing certain colours if they have an intensity above a certain pre-defined value.

The images captured by the CCD-camera are divided into pixels. The intensity of each pixel is then calculated. A threshold value is used to differentiate between the neoplastic tissue and normal tissue. To obtain this threshold value a reference marker is searched for to benchmark the value for normal tissue compared to the neoplastic tissue. The data are then normalised with respect to the reference marker.

1.5.2 Autofluorescence

Endogenous fluorescence or autofluorescence is based on the normal fluorophores that exist in the body and skin. Well known endogenous fluorophores are elastin, collagen, NADH,

keratin and tryptophan [91-93]. Any given tissue has a mixture of many variable fluorophores and they are not uniformly distributed in the tissue.

The architecture in normal and diseased tissue differs both in structural and metabolic composition[85] . Examples of metabolic fluorophores are NADH-NAD+ and flavins and examples of structural fluorophores are collagen and elastin crosslinks in the dermal matrix. In NMSC such as BCC and SCC, Brancalean *et al.*[85] discovered in 2001 that endogenous fluorescence from tryptophan-residues was more intense in tumours due to tumour epidermal thickness or proliferation or both. Less fluorescence was seen from dermal collagen crosslinks, probably due to degradation or erosion of connective tissue, due to enzyme degradation from tumours. Therefore it was possible to selectively separate normal tissue from tumour tissue. When investigating autofluorescence the emission spectrum is in the green spectrum and is separated from the ALA-FD whose emission is in the red spectrum.

1.5.2 ALA-induced fluorescence

ALA or MAL is utilized in the same manner in FD as in PDT. Similarly to PDT, a 3h application time of the pro-drug is most commonly used to obtain as high concentration as possible within the tumour tissue while simultaneously avoiding any accumulation of PpIX-concentration in the surrounding normal tissues. This is to ensure the contrast between the different tissues is as large as possible [49]. It is necessary to receive as high a contrast value as possible between the neoplastic tissue and normal tissue to ensure that high resolution is achieved in the images [94]. PpIX absorbs intensely at the Soret-band (380-420nm) with a peak at 405 nm (blue light) and fluoresces at round 630nm (red light). Thus UV-blue light is used as an excitation light source and causes the PpIX to fluoresce red light. A filter in front of the CCD-camera selects this wavelength and a fluorescence image is captured, see **figure 5**. This

image can be further analyzed to obtain additional information. A study of mouse skin by Moan (2001) [47] has previously suggested that MAL is more selective than ALA. de Bruijn *et al.* [95] examined mouse skin with fluorescence microscopy in 2008 and found no difference between the two pro-drugs.

1.6 Microdialysis

Microdialysis (MD), is a method that has been widely used during the last decades. The method is based on passive diffusion. Low molecular weight substances passively diffuse through the semi-permeable membrane along a concentration gradient of compounds in extra-cellular fluid. A small diameter probe containing a dialysis membrane is implanted into tissue and perfused with a suitable fluid. The catheter membrane has a molecular weight cut-off of a certain molecular size and therefore molecules smaller than this will be able to diffuse across the membrane into the perfusate.

This technique was first applied in animal studies but later also on humans primarily to study brain function and changes in levels of endogenous compounds such as neurotransmitters or metabolites. The development of MD for the purpose of measuring drugs was initiated during the late eighties. Nowadays almost all tissues have been investigated, including brain tissue, skeletal tissue, muscles, subcutaneous adipose tissue, tendons, and skin among others [96,97].

1.6.1 Microdialysis in the skin

The MD technique can be applied for assessing percutaneous absorption in the skin[98]. It measures compounds in extra-cellular fluid over a semi-permeable membrane/filter of an MD-probe. The microdialysis catheter is guided into the dermis through a needle, thereafter the needle is removed with the catheter thus in place. To imitate the blood flow the catheter is connected to a precision pump which slowly perfuses it with a tissue-compatible sterile buffer

solution, see **figure 1, page 3, of Paper III**. Samples of the perfusate will be assessed for analysis at specified time intervals[99]. A problem with the insertion of the probe into the tissue is that it will elicit a tissue reaction, the flare reaction, causing changed skin circulation, which in turn can influence the skin metabolism[100]. Only a fraction of the substance will be recovered in the dialysate. The recovery depends on many factors, such as how long after the flare samples were collected, the perfusates' properties, where and how the probe is situated and the substance qualities. In addition the MD-technique is not very suitable for lipophilic or highly protein-bound chemicals due to the small recovery[101,102].

The lipophilic substance can be trapped in the *stratum corneum* and the highly protein-bound complex will often be too large to pass across the semi-permeable membrane.

1.6.2 Calibration of the microdialysis technique

Several articles have established a way to calibrate the MD-method and calculate the recovery[103]. The basis of the calculation is the assumption that the glucose or urea level is the same in the extra-cellular fluid as in the blood-plasma and that the *in vivo* recovery ratio for glucose or urea is the same as for the substance[104], for example ALA, to be sampled. Then the relative recovery and the interstitional concentration of ALA(= I) can be calculated as:

$$\frac{RR(ALA)_{in\ vitro}}{RR(Urea)_{in\ vitro}} = \frac{RR(ALA)_{in\ vivo}}{RR(Urea)_{in\ vivo}}$$

$$RR(Urea)_{in\ vivo} = \frac{\text{Urea dialysate concentration}}{\text{Urea plasma concentration}}$$

$$I = \frac{\text{ALA dialysate concentration}}{RR(ALA)_{in\ vivo}}$$

2. AIMS OF INVESTIGATIONS

The investigations conducted as part of the thesis have had the following aims:

- to evaluate the impact of the fluence rate and spectral range on PDT for AKs
- to register the distribution of pain during PDT of AK
- to investigate which factors are related to pain during PDT of AK
- to investigate the tumour penetration and interstitial concentration of ALA and MAL following topical application in BCCs *in vivo* using microdialysis
- to evaluate the fluorescence contrast in patients treated with MAL-PDT for superficial BCCs and to compare the use of MAL with ALA as the pro-drug.
- to find out whether poorly responding BCCs lack accumulation of PpIX after ALA and MAL application

3. PATIENTS AND METHODS

3.1 Papers I-IV

Paper I

Patients

A total of 40 patients, with clinically typical AKs, were included in an open, multi-centre, randomised study. Three patients were excluded from the analysis: one due to the lesion being diagnosed as a SCC and two due to equipment failure. In total 35 of the 37 lesions were histologically verified (32 men and 5 women, mean 71).

Method

Evaluation of the lesion and primary treatment outcome

The AKs in the treatment area were drawn on a plastic film and the percentage of the AKs in relation to the total treatment area was calculated.

Application of ALA cream

ALA cream of 20% w/w δ-5-ALA-HCl in Unguentum-Merck® was applied in an approximately 1 mm thick layer for 3 h. A special occlusive bandage (Medeikonos AB, Sweden) 5 cm × 5 cm was used.

PDT and fluorescence imaging

PDT and FD equipment consisted of a non-coherent mercury lamp, Photodemarcation system 1, Prototype 5 (Medeikonos AB, Sweden) with one arm for PDT and the other for FD. The arm for PDT was equipped with broad and narrow band filters. The broad band had a spectral range of 580 - 690 nm and the narrow band had a spectral range of 580 - 650 nm. The narrow filter produced two groups of fluence rates: 30 and 45 mW/cm^2 (N30 and N45). The broad band filter also produced two groups of fluence rates: 50 and 75 mW/cm^2 (B50, B75).

The dose for all treatment groups was 100 J/cm^2. The effective fluence rates were 9.6 mW/cm^2 (N30), 15 mW/cm^2 (N45/B50) and 22 mW/cm^2 (B75) (see 1.4.5 Dosimetry). The light for FD was concentrated to two peaks: 365 nm and 405 nm (UV-blue spectra), with

a fluence rate of 0.5 mW/cm^2. The PDT was interrupted for fluorescence imaging at the accumulated doses of: 5, 10, 20, 40 J/cm^2 and after the total dose of 100 J/cm^2.

Pain assessment

After every interruption of the PDT-treatment, the patients were asked to record their maximum pain experienced during the previous PDT-session. The VAS-scale was graded from 0 (no pain) to 10 (worst pain imagined).

Analysis of bleaching data

Reference markers were used for normalisation. All data were collected from a cross-section including the reference markers and the AKs. This was completed for every image. The data were normalised using the reference markers. The cross-section data were then divided into 5-10 regions. A mean value for every region was calculated. The mean value of bleaching data from all the 5-10 regions was calculated for each patient. Three patients were excluded from the analysis due to lack of reference markers and the inability to normalise the data from these images. A fourth patient was identified as a suspected outlier This patient was also excluded from the analysis.

Follow-up

After 7 weeks a follow-up visit was performed with an evaluation of the treatment response and remaining AKs were noted.

Paper II

Patients

An open, multi-center, study including 94 patients with AKs (70 men and 24 women), ranging from 52-91 years of age with a mean of 76, were investigated to register pain levels experienced during PDT. All AKs were histologically confirmed. Three patients were excluded from the analysis, as two showed BD histologically and a third due to technical problems during the treatment. Therefore, 91 patients were deemed suitable for evaluation in

the study. Size, redness, scaling and induration were recorded. Maximum pain during treatment was measured using VAS as described previously.

An additional pilot study was conducted on 7 patients (5 men and 2 women), of ages ranging between 50-81 years, mean 65, were included to test the pain reducing effect of pre-treatment with capsaicin.

One patient discontinued the study due to local side-effects. Therefore 6 patients with a total of 10 lesions (1BD, 1superficial BCC, 8AKs) were deemed suitable for further analysis.

Methods

Photodynamic treatment

ALA cream of 20% w/w δ-5-ALA-HCl in Unguentum-Merck® was applied in an approximately 1 mm thick layer over a 5 cm × 5 cm treatment area for 3 h under occlusion. Red light (590-650 nm) from a non-coherent light source (Photodemarcation System 1, Prototype 5 Medeikonos AB, Göteborg, Sweden) was used (dose 70J/cm^2 and fluence rate of 70 mW/cm^2). Complete response was defined as clinically 0-1% remaining AK.

In the pilot study, pre-treatment application with capsaicin cream on the treatment area was applied 3-5 times per day for 1 week before commencing PDT. Red light (600-730 nm) with a PDT 1200 lamp (Waldmann Medical, Schwenningen, Germany) was used**.**

Pain registration

The pain was registered using VAS. The scale was divided into 3 groups: low or no pain, VAS 0-3; moderate pain, VAS 4-6; and severe pain, VAS 7-10. Fans and cold water spraying was offered, but no anesthesia was given during the treatment.

Paper III

Patients

Twenty patients (4 women and 16 men), of age ranging between 51-93 years, mean 70, with 27 BCCs (13 superficial, 14 nodular) were included. All lesions were histologically verified. The first ten patients were treated with MAL (13 BCCs, 8 nodular and 5 superficial), and the following ten patients received ALA (14 BCCs, 6 nodular and 8 superficial). Every second lesion was pre-treated with a light curettage (curettage: n=13, non-curettage: n=14).

Methods

Drugs

20% w/w 5-ALA cream (in Unguentum-M®, Hermal, Reinbek, Germany) and 16% methylaminolaevulinate-cream (Metvix®, Photocure, Oslo, Norway) was applied to the BCCs in a 1 mm thick layer and then occluded for 3 h.

Microdialysis procedure

With guidance from a needle inserted intracutaneously, in or slightly under the BCCs, microdialysis catheters were inserted into the tumours at tissue depths varying between 0.4 to 1.9 mm. The depth was measured with ultra-sound. A precision pump constantly perfused

physiological NaCl solution at a rate of 2 µl/minute. Dialysates were collected at 15-30 min intervals for 4 h.

Chemical analysis

The interstitial concentrations of MAL and ALA were determined by the certified clinical chemistry laboratory at our university hospital using high-performance liquid chromatography (HPLC). A routine method for the determination of amino acid concentrations was used. The concentrations of ALA, MAL and 24 endogenous amino acids were determined for each sample.

Calibration

To estimate dermal interstitial ALA and MAL concentrations, the concentrations of microdialysed ALA and MAL were determined *in vitro* in blood plasma, to which three known concentrations of ALA and MAL were added. The *in vitro* recoveries of ALA and MAL were then calculated and found to be 4 ± 1 % and 6.3 ± 1.5 % respectively, see section 1.6.2.

Paper IV

Patients

Twenty-four patients, (13 male and 11 female) with ages ranging between 36-86 years, mean 67, with one or more clinically suspected superficial BCCs, were included in this open prospective study. Two of the patients discontinued prematurely. Twenty-two patients with a total of 35 lesions were included in the analysis.

In a previous study by our group, the fluorescence of BCCs treated with ALA was investigated on forty patients with a total of forty lesions using the same fluorescence imaging device, as described below [49].

Methods

Drugs

The MAL cream (160 mg/g, Metvix®, Photocure, Oslo, Norway) was administered to the tumour in an approximately 1 mm thick layer, including at least a 1 cm surrounding margin, and occluded for 3h.

PDT-procedure

The lesions had two sessions of PDT performed approximately one week apart with red light (approximately 635 nm, a fluence rate of 37 mW/cm^2 and a total light dose of 37 J/cm^2) using an LED-lamp (Actilite®, Photocure, Oslo, Norway).

Assessment of pain

The patients were asked to state the maximum pain experienced during both treatments using a visual analogue scale (VAS), ranging from 0 (no pain) to 10 (unbearable pain).

Fluorescence diagnostics

Fluorescence imaging was captured on all lesions immediately before the two sessions of irradiation. A Photodemarcation System 1, Prototype 5, (Medeikonos AB, Gothenburg, Sweden) with main excitation emissions of two peaks around 365 and 405 nm, having a fluence rate of 0.5 mW/cm^2, was used. The detector consisted of a filtered CCD-camera with fluorescence emission in the range of 610–715 nm, matching the PpIX emission at 635 nm.

Follow-up

The treatment outcome was assessed as: completely cleared, partially cleared (approximately 50%) or not cleared, at a visit 7-18 weeks after the second treatment. Any adverse events were recorded.

Image analysis

On the digital clinical photographs, the clinical margins of the BCCs were marked by a clinician. The tumour margins in the fluorescence images were collected using a semi-automated threshold algorithm implemented in Matlab®. After extracting the areas corresponding to the tumour area (T), normal MAL treated skin (N) and baseline (B) using the semi-automatic segmentation program, the mean intensity (I) in the selected regions was calculated. Thereafter, the fluorescence contrast, defined as:

$$\kappa = I_T/I_N$$

could be calculated. The area of the detected tumour region was also compared to the clinical tumour markings and their correlation was assessed.The mean fluorescence contrast obtained, after a 3 hour application of ALA, was calculated. In our earlier study on ALA [49], we calculated a peak contrast value including only 10% of the highest fluorescent pixels within the marked tumour area of all tumours.

3.2 Statistics

Paper I

To find out whether there were any differences between the groups, ANOVA and Student's *t*-test were used, two-tailed tests were applied and, to find potential outliers, the Dixon's Q-test was utilized. To test for correlations, contingency tables were applied.

Paper II

To examine differences between two groups, independent *t*-test procedure was used. When comparing more than two groups, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used. To analyse population distribution a Jarque Bera test using Matlab® Statistics Toolbox was applied.

Paper III

Student's two-sample *t*-test, Fischer's test and correlation coefficient methods were used. Mann Whitney *U*-test was applied to test for differences in distribution between groups.

Paper IV

Student's *t*-test and Wilcoxon's signed rank test were used as statistical methods. Pearson's correlation coefficient and Spearman's *rho* were used to test for correlations. Three fluorescence images were excluded from the analysis as the selected ROI were found to contain areas with field cancerization and actinic keratosis. Fluorescence contrast data are presented as mean values ± standard error of the mean (SEM)

3.3 Ethics

Paper I

The study was approved by the Ethical Committee of Linköping University and was performed at the Departments of Dermatology at Sahlgrenska University Hospital (Gothenburg, Sweden), Karolinska Hospital (Stockholm, Sweden) and Linköping University Hospital (Linköping, Sweden).

Paper II

The local Ethics Committee of Linköping approved the study and the local Ethics Committee of Gothenburg approved the pilot study with capsaicin. Ten centres recruited the patients in the main study. All patients in the pilot study were recruited in Gothenburg.

Paper III and Paper IV

The studies were approved by the local Ethics Committee and performed at the Department of Dermatology and Venereology at Sahlgrenska University Hospital, Gothenburg, Sweden.

4. RESULTS

4.1. Papers I-IV

Paper I

When comparing the 4 groups with different spectral emissions and fluence rates, it was found that the group with the narrow filter and 30 mW/cm^2 (N30) had higher number of patients with complete remission compared to the other groups.

The treatment outcome and fluence rate were found to correlate ($p<0.02$). The rate of photobleaching was also higher for the N30 group showing that the photobleaching is dependent on the fluence rate (see **Table 2, p.1208, paper I**). No significant difference was seen between the N45 and the B50 group with the same effective fluence rate (see section 1.4.5 for definition), suggesting that it is the fluence rate and not the spectral range that is significant. No further photobleaching could be detected by the eye after a dose of 20 J/cm^2, but when calculation was performed by the computer, slightly more photobleaching could be identified up to the dose of 40 J/cm^2. No correlation between the fluence rate and pain could be observed, but it was observed that the pain increased up to 20 J/cm^2 and then gradually decreased during the treatment up to 100 J/cm^2 (see **figure 5a, p. 1209, Paper I**).

Paper II

It was noted that the pain was normally distributed ($p=0.34$) around a mean VAS value of visual analogue scale 4.6 although there was a large intervariation between the patients (see **figure 1, p.405, Paper II**). While there was a large group (31%, n=29) with no or low pain (VAS 0-3), a not negligible group (21%, n=19) had severe pain (VAS 7-10). Risk factors for pain were, larger lesions ($p=0.001$, see **figure 2, p. 406, Paper II**), the redness of the actinic lesions ($p=0.01$), and the reduction of the actinic area ($p=0.007$). Redness was also correlated

to the cure rate (p=0.01). The redder the actinic area, the better the treatment outcome and the more pain experienced. Patients with the largest reduction in the actinic area experienced more pain (p=0.053). A trend was noted showing a more severe experience of pain in patients with skin type I-III (n=88) compared with those with skin type IV (n=3). However no statistical significance was reached (p=0.058).

For those patients pre-treated with capsaicin no significant pain relief was noted.

Paper III

The location of the catheter depths in the two groups (ALA and MAL) was not found to be significantly different (P>0.18), although the depths for the ALA-catheters ranged from 0.4 to 1.4 mm (n=14), and the MAL-catheters from 0.6 to 1.9 mm. During the 4 hour measurement period we could not find any significant difference in interstitial drug concentration between lesions treated with ALA or MAL. The mean concentration reached a plateau level after 15 minutes. However, of 11 lesions in which the microdialysis catheter was located at a depth below 1 mm, drug concentrations above the detection limit were only obtained in 6 lesions, while 15 of 16 BCCs with a more superficial catheter location (i.e. less than 1 mm deep) exhibited detectable drug concentrations (p=0.026). Detectable levels of the drug were not obtained in almost 50% of the lesions where catheters were situated 1 to 1.9 mm in the lesion. The extra-cellular peak concentrations were reached within 90 minutes in 23 of the 27 BCCs, but were not found to be correlated with the depth of the catheters. Surprisingly the effect of curettage was found negligible and no difference could be identified between superficial and nodular BCCs.

Paper IV

The detected tumour area of superficial BCCs from the fluorescence images was found to be larger than the clinical border if the clinical tumour was less than 1 cm^2, (see **figure 3, p.26,**

Paper IV). No significant difference in tumour area was observed when comparing the fluorescence images obtained before the first and second PDT sessions, (see **figure 4, p. 27, Paper IV)**. As expected, the contrast between the MAL-treated normal skin and the non-treated normal skin was significantly lower than the contrast between the MAL-treated tumour and MAL-treated normal skin. When comparing the contrast reached from the first and second treatment, this increased from 1.65 ± 0.06 to 1.84 ± 0.07 ($p< 0.01$). As fluorescence images from BCCs treated with ALA from a previous study[49] were also available, it was possible to make a comparison between MAL and ALA. The mean fluorescence contrast obtained during the first treatment with MAL (1.65 ± 0.06) was significantly higher than the mean fluorescence contrast obtained after a 3 hour application of ALA (1.20 ±0.06) ($p<10^{-4}$), (see **figure 5, p. 28, Paper IV)**. In our previous study on ALA[49], we calculated a peak contrast value including only 10% of the highest fluorescent pixels within the marked tumour area of all tumours. The value of the peak ALA contrast was found to be 1.61 ± 0.10. This value is similar to the mean contrast value obtained with MAL. Thus the mean contrast with MAL is as high as the peak contrast obtained with ALA.

The cure rate was calculated at our follow-up visit, 7-18 weeks after the last treatment. We found that 91% (n=32) were completely cleared, 6% (n=2) were partially cleared and 3% (n=1) did not respond to PDT. We also determined the fluorescence contrast and compared the completely cleared tumour area with the partial or non-cleared areas and found no correlation between the fluorescence and lack of treatment response. However, due to the small number of patients not responding to treatment in this study, no statistical analysis could be made. It was not possible to correlate the fluorescence obtained with the measured VAS (data not shown).

5. DISCUSSION

5.1. Methodological considerations

Paper I

Fractionation of PDT may affect the treatment outcome. In our study the PDT was arrested for approximately 2 minutes between every dose investigated. During this time, the amount of PpIX would not have increased to a great extent since conversion to PpIX requires a greater period of time. The tissue would to some extent have been re-oxygenated in the break but this was the same for all fluence rates.

A cross-section was analysed and not the whole area treated which might influence the results to some degree: but, since this methodology has been used to analyse all data, the relationship between the groups and results would only be marginally affected.

In the study presented here all areas were calculated by hand which would introduce a small uncertainty.

Fluorescence measures only the superficial PpIX and gives a measure of the amount deeper in the lesion. Our results may not be the true for the PpIX below surface levels.

Paper II

In the main study the field treated was small (5 cm × 5 cm). This area matched the light source treatment field which was available at the time when the study was performed. Taking into consideration that there are now light sources with treatment areas of 10 cm × 20 cm, this might affect the results of the assessment of the pain induced by the treatment if the study was re-run today, i.e., larger treatment areas tend to give larger pain.

The pilot study aimed to analyse whether capsaicin had any pain reducing effect during illumination. All participating patients experienced the expected adverse events of erythematic skin and burning sensation in the treatment area after applying the capsaicin cream at home. Because of the pain experienced during application, one may question patient

drug compliance, which may have affected the final study result. The question was raised of extending the pre-treatment time with capsaicin to two weeks; but, the effect of this is questionable as the expected patient compliance due to pain experienced is low. The study would have been improved if we had selected patients with two areas needed to be treated at one time with one area receiving the cream and the other placebo-cream in a comparative study.

Paper III

The number of patients was limited due to the inconvenience to participate. All patients were required to lie down on their back for 8 hours during the measurements as all lesions studied had to be located on the front of the body.

Calibration was made from blood plasma supplied from the hospital's blood bank. The method may have been improved if the recovery had been calculated by analyzing an amino–acid both from the blood and extra-cellular fluid directly from the patients' plasma.

There were difficulties with the insertion of the micro-dialysis catheters and to know in advance at what depth it would be inserted. The maximal catheter depth for BCCs treated with ALA was 1.4 mm and the maximal catheter depth for lesions treated with MAL was 1.9 mm. The intention was of course to have the two groups as similar as possible.

We could perhaps have used PDT to treat all patients, in order to be able to correlate the treatment outcome with the concentrations reached. However due to the inclusion of so many nodular BCCs, we chose to treat the BCCs according to recommended guidelines.

Paper IV

The clinical borders of the BCCs were marked on the digital photographs and not directly on the patients. It would have been easier to have marked the borders of the BCCs on the patients, but since we had performed a previous study where the borders were marked on the patients using a black ink pen and this disturbed the fluorescence images marking on the photos was chosen to minimise disturbance of the fluorescence images.

Fluorescence diagnostics only measures the superficial PpIX and does not show the amount of the PpIX in the deeper parts of the BCCs. If we had included thin nodular BCCs it may have been easier to evaluate any differences between the PpIX accumulation in the "difficult to treat" BCCs.

In retrospect it might have been better to collect the ALA-data and the MAL-data at the same time. However as the equipment was used for the same procedures and the raw images were available from the previous study we concluded that this was not a major issue.

5.2. General discussion

PDT is a widely used method for a number of diagnoses today, some with excellent results such as thin NMSC like Bowen's disease, AKs and superficial BCCs [105]. These results are due to the right diagnosis for treatment but also to the use of the best treatment schedule. However, the method still needs to be refined to minimise unwanted side effects like pain, and the pre-treatment time with ALA and MAL which is inconveniently long. The cure rate for, e.g., nodular BCCs is somewhat low unless extensive debulking is used which then will interfere with the good cosmesis, which is one of the advantages of the method.

Fluence rate, photobleaching and dose

There are different treatment regimes for different light sources but also due to traditional hospital therapies. In the study in **Paper I** the photobleching was measured up to the dose of 100 J/cm^2, showing that doses above 40 J/cm^2 did not demonstrate increased photobleaching, i.e., all PpIX was depleted. Thus, it seems unnecessary to use doses above 40 J/cm^2 (635 nm, non-coherent light, Photodemarcation System 1). When reducing the dose to 40 J/cm^2 with a low fluence rate, the treatment time could be decreased resulting in less pain.

We have demonstrated that it was favourable to use lower fluence rates for the treatment outcome as well as positively influencing the photobleaching. This seems to be explained by the oxygen depletion rate, which is slower with low fluence rate[59]. We showed that lower fluence rates resulted in lower bleaching doses. The bleaching dose was found to vary between the patients despite the fact that they were treated with the same fluence rate. The explanation for this might be that external factors are involved. For example, some patients are more anxious and may move during the treatment. The vascularisation is individual in all the patients as is the skin temperature which has earlier been shown to be influencing the PDT-treatment[57].

During the photobleaching of PpIX, formation of photoproducts takes place. It has been discussed whether these photoproducts are involved in the PDT process or not [106]. It has also been speculated whether these photoproducts are involved in the pain or not. In the first study we included two types of spectral ranges. The broad spectrum included the absorption by the photoproducts around 670 nm, while the narrow spectrum excluded this absorption peak. The results from **Paper I** did not show any influence of the spectral range on either the pain, photobleaching or primary outcome.

Pain

The pain peak during PDT was reached early, i.e., within seconds or minutes and after 20 J/cm^2 it gradually decreased. After completion of the light therapy, the pain decreased radically (”like switching off a light”). Since later studies have showed that the pain is correlated to the PpIX-accumulation [69], the decrease of the pain might be explained by the fact that after the dose of 20 J/cm^2, only a small part of the PpIX is left to photobleach. In this study, the photobleaching was high in the beginning of the treatment, but after the dose of 10 J/cm^2 the photobleaching rate decreased. Even if we cannot entirely explain the things that

take place, knowledge of the pattern is an important factor when informing the patient about the treatment and the known pain response, thereby bestowing a sense of security on the patient.

Pain is the main disadvantage of PDT; other disadvantages are of minimal importance. During recent years research has focused on pain and pain-relieving strategies. Pain during PDT can sometimes be so severe that the patients either interrupt the treatment or endure the pain experiencing severe agony. It is therefore important to learn more about pain response during PDT.

In **paper II**, pain experienced during PDT of actinic keratoses was investigated and we could confirm the results from our previous results, i.e., that pain is normally distributed in a Gauss curve[68]. Of 91 patients included in the study, approximately 30% experienced VAS 0-3 while as many as 20% of the patients experienced VAS 7-10. This indicates the importance of identifying our search for better pain relieving strategies. We also confirmed that larger lesions gave more intense pain (Grapengiesser *et al.*[68]).

A unique finding was a direct relationship between the redness, i.e., inflammation and vasodilatation of the lesion, which lead to increased pain but also improved treatment results. One theory is that in a vasodilated area available oxygen is increased which may lead to a better penetration of the pro-drugs. Furthermore, inflammatory diseases have also been treated with PDT with some effect, indicating that it may not only be the neoplastic cells which respond to treatment but also the inflammatory cells [107]. Thus a larger number of cells will be damaged and may cause greater pain than when only the neoplastic cells are treated. Morton has previously mentioned his theory of “no pain, no gain”. In our study we found that patients

with the largest reduction in the actinic area experienced more pain (p=0.053), in support of Morton's theory.

To our disappointment, the capsaicin treatment as a pain reliever was not useful. Previous studies have shown that not only substance P is depleted but also that the nerve-endings are affected histologically after only 3 days of treatment with capsaicin at the same concentration (Wallergren and Håkansson [108]). Nolano *et al.*[109] performed a study, using capsaicin 0.075%, and showed a significant degeneration of nerve-fibres within a few days. One explanation for the lack of effect of the capsaicin treatment was that the compliance was not good and could have interfered with the results. This would certainly explain our findings but not exclude the need for more study to confirm the function of capsaicin. The patients included had previously experienced very severe pain during treatment, thus indicating the necessity for a stronger pain reliever. If patients with moderate pain had been included in the study it may have shown that the pain reliever could have had some effect.

Difficulties with PDT

There is an ongoing discussion concerning whether nodular BCCs are suited for PDT and how the pre-treatment should be performed. Previous studies have shown that the cure rate for nodular BCCs is lower than for superficial BCCs [38,110-112]. Furthermore, one of the advantages with PDT is that it provides excellent cosmetic outcome. If extensive debulking is required for a tumour to be completely removed this will significantly affect the cosmetic outcome.

The comparison between the two pro-drugs ALA and MAL is also interesting. In **Paper III,** we investigated both superficial and nodular BCCs and measured the amount of pro-drug required both with and without curettage. We could not find any significant difference in

concentration between the two pre-treatments. It should be noted that this is not in accordance with some previous results as described below.

Van den Akker described in a study in 2003[43] that tape-stripping of normal nude mouse skin increased the PpIX-concentration implying that *stratum corneum* is an important barrier for the pro-drugs. Furthermore a study by Gerritsen[44] and one by Smits *et al.*[45] showed a negative correlation between thickness of *stratum corneum* and the amount of superficial PpIX measured indirectly by fluorescence diagnostics. One should note, however, that the diagnoses investigated were: normal skin, keratinocytic intraepidermal neoplasia, verrucous hyperkeratoses, actinic keratoses, i.e., *not* BCCs.

Indeed our results are in accordance with a recent study performed by Moseley *et al.*[46] who investigated superficial BCCs and BDs and who found no significant difference between tumours who had received curettage and those who had not, neither in measured superficial fluorescence nor in cure rate.
One should keep in mind, however, that there may be an important difference due to the different diagnoses as in cases where the barrier for BCCs has already been broken there may be no need for pre-treatment.

We found no difference between the transdermal penetration of ALA and MAL. A plateau level of concentration was reached after 15 minutes. This is in agreement with another microdialysis study performed by Wennberg *et al.*[42], where the rapid attainment of a plateau level of the concentration was noted. However, the measurements were performed no deeper than 0.5 mm.

In our microdialysis study, **Paper III,** the location of the catheters were measured by ultra-sound to a depth between 0.4 - 1.9 mm. There was no problem with the penetration of the pro-drugs to a depth of 1 mm. However, between 1 - 2 mm of depth, only about 50% of the measured lesions showed any concentration at all of the pro-drugs. If no concentration can be assessed, the question to be asked is "what will the cure rate be in those lesions"?

There is pressure to have the pre-treatment time reduced, as the standard time for MAL is 3 hours[49]. Lately Braathen *et al.*[50] investigated PDT on AKs and used two groups of incubation times with MAL: 1 hour and 3 hours. The cure rate decreased slightly in the first group but the practical benefit of the shorter incubation time may make this treatment attractive. In our microdialysis study, we found that 23 out of 27 BCCs, reached their peak concentration within 90 minutes, but this was shown via measurements of the pro-drug in the extra-cellular space and not the PpIX within the cells. The results may still be an indication that shorter pre-treatment time of the pro-drugs would not significantly effect the outcome of the treatment.

Finally, we did not find any difference in penetration of the two pro-drugs between superficial and nodular BCCs. This is an indication that there is no tissue dependent difference between the penetrations of the pro-drugs. Instead it was discovered that the thickness of the BCC is very important. Most nodular BCCs are thicker than the superficial BCCs and this may be one explanation for the somewhat lower cure rate for the nodular BCCs.

Fluorescence diagnostics

Very few studies of FD have been performed comparing the pro-drugs ALA and MAL. One study in mouse skin by Moan *et al.*[47] found that the ALA-induced fluorescence was visible

outside the topical treatment field while MAL-induced fluorescence was only seen in the treated field.

In the final study presented within this thesis (**Paper IV**) we have analysed 37 BCC lesions on human skin *in vivo,* treated with MAL and compared it with a previous study using ALA as a pro-drug on 40 BCCs (also conducted on human skin *in vivo)* with the same equipment and procedure. Interesting data from the MAL-treated BCCs were obtained, evaluating the first and second PDT where the mean contrast increased from 1.65 ± 0.06 to 1.84 ± 0.07 at the second treatment ($p< 0.01$).

This can most probably be explained by an increase in the penetration of the pro-drug, i.e. MAL, into the tumour due to disruption of the penetration barrier after the first treatment. This effect of PDT on the skin barrier function has previously been confirmed in rodents[95]. Another possible explanation for the higher PpIX accumulation in the tumour area at the second PDT session might be the presence of inflammatory cells as a result of the first treatment. Inflammatory cells have been reported to show high accumulation of PpIX after administration of ALA or MAL Thus, the presence of inflammatory cells at the second PDT session could contribute to the higher accumulation of PpIX in the tumour region[95].

The mean fluorescence contrast obtained during the first treatment with MAL (1.65 ± 0.06) was significantly higher than the mean fluorescence contrast obtained after a 3 hour application of ALA (1.20 ±0.06). In the published ALA-study[49], we calculated a peak contrast value including only 10% of the highest fluorescent pixels within the marked areas, instead of a mean value, and this was found to be 1.61±0.10. This value is similar to the mean contrast value obtained with MAL. Thus, the mean contrast with MAL is as high as the peak contrast obtained with ALA. This implies that when performing FD, MAL is the treatment of choice.

This might be due to the fact that MAL, in contrast to ALA, is more selective and has only one pathway to be distributed into the tissue. Therefore it could take longer time before there will be any MAL outside the tumour area.

6. CONCLUSIONS

Both the photobleaching rate and primary treatment outcome are dependant of fluence rate. A low fluence rate (30mW/cm^2) seems preferable when performing PDT of AK using non-coherent light sources. No influence of spectral range was seen, on the pain or primary treatment outcome

The pain score was normally distributed around a mean value of VAS 4.6.

The most important factors effecting pain during PDT seem to be the size and the redness of the lesion.

No significant difference between the tumour penetration ability of ALA or MAL was found. Detectable levels of the drugs were obtained in only 50% of the lesions where catheters were placed > 1 mm deep. A pre-treatment curettage did not affect drug concentration. Thus penetration of the drug is uncertain when treating BCCs thicker than 1 mm.

The fluorescent tumour area in small BCCs (<1 cm^2) was larger than the clinically marked tumour. The mean tumour contrast was increased from 1.65 ± 0.06 at the first treatment, to 1.84 ± 0.07 at the second treatment ($p < 0.01$). MAL renders a higher tumour contrast compared to ALA in superficial BCCs.

No correlation between fluorescence and lack of treatment response could be observed.

7. OUTLOOK FOR THE FUTURE

Both PDT and FD are relatively new techniques and need to be improved.

Since there are difficulties with the penetration of the pro-drugs in the deeper regions of the tumours, new derivates, new delivery systems and transport media for the pro-drugs need to be found to increase penetration. Better delivery formulations could be cubic lipids [113,114]. These would be interesting to test using the MD-technique.

The pre-treatment time with ALA and MAL is too long to be convenient. If the delivery systems are changed further studies with shorter pre-treatment time would be interesting to perform also for other diagnoses than AK (Braathen[50]). Further investigations are necessary to calculate the time needed for the pro-drugs, ALA and MAL, to penetrate the lesions and to accumulate enough PpIX to successfully conduct PDT. This could possibly be tested by fluorescence microscopy on biopsies.

A new ALA-patch delivery system [115] has been placed on the market, with the advantage that the patients do the pre-treatment at home and only enter the hospital for the irradiation with red light. Maybe with sufficient instruction the patients could also apply the cream with the pro-drug at home, making the inconvenience of the long pre-treatment time less.

No further photobleaching could be detected by the eye after 20 J/cm^2, but when calculation was performed by the computer a slightly increased photobleaching was detected up to 40 J/cm^2, but this is not the effective light dose.
Today the standard dose for treating with an LED-source is 37 J/cm^2. Since LED has a narrower spectrum than the lamp we used it is possible that we are over-treating these patients when treating superficial (pre)tumours like AK and SBCC. When considering the adverse side-effects such as pain, the reduction of the dose by half without interfering with the cure rate would lead to the treatment time being reduced to half. This would be an interesting study to perform.

The main disadvantage of PDT is *pain*. There have been many studies performed focusing on finding effective pain reducing treatments. The most effective treatment has recently been shown to be nerve blocks for pain relief of the face and scalp (Paoli *et al.*, 2008 and Halldin *et al.*, 2009) This treatment is, however, only available for the scalp, forehead, chin and medial cheek. For PDT to survive as a method of treatment in other areas of the body it is necessary to discover a successful pain relief treatment. A study designed to find new specific areas of the skin where peripheral nerve blocks maybe used is appealing.

There is a need to improve the FD-method as it is not yet fully acceptable. Further study of the effect of combining the autofluorescence of BCC with the MAL-induced fluorescence, to increase the safety of FD use as a pre-surgical tool, would be a great asset and to use Mohs' surgery to do control-mapping.

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REFERENCES

1 Kanitakis J. Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol* 2002; **12**: 390-9; quiz 400-1.
2 Rorsman H, Björnberg A, Vahlquist A. [The skin's structure and function]. *Dermatologi Venereologi* 2000; Lund: 13-30.
3 Yamaguchi Y, Brenner M, Hearing VJ. The regulation of skin pigmentation. *J Biol Chem* 2007; **282**: 27557-61.
4 Dessinioti C, Stratigos AJ, Rigopoulos D et al. A review of genetic disorders of hypopigmentation: lessons learned from the biology of melanocytes. *Exp Dermatol* 2009; **18**: 741-9.
5 Ibbotson SH. Topical 5-aminolaevulinic acid photodynamic therapy for the treatment of skin conditions other than non-melanoma skin cancer. *Br J Dermatol* 2002; **146**: 178-88.
6 Cordoba F, Braathen LR, Weissenberger J et al. 5-aminolaevulinic acid photodynamic therapy in a transgenic mouse model of skin melanoma. *Exp Dermatol* 2005; **14**: 429-37.
7 Fuchs A, Marmur E. The kinetics of skin cancer: progression of actinic keratosis to squamous cell carcinoma. *Dermatol Surg* 2007; **33**: 1099-101.
8 Scurry J. Grading of actinic keratoses. *J Am Acad Dermatol* 2001; **44**: 1052-3.
9 Armstrong BK, Kricker A. The epidemiology of UV induced skin cancer. *J Photochem Photobiol B* 2001; **63**: 8-18.
10 Hansen JP, Drake AL, Walling HW. Bowen's Disease: a four-year retrospective review of epidemiology and treatment at a university center. *Dermatol Surg* 2008; **34**: 878-83.
11 Brantsch KD, Meisner C, Schonfisch B et al. Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. *Lancet Oncol* 2008; **9**: 713-20.
12 Sabin SR, Goldstein G, Rosenthal HG et al. Aggressive squamous cell carcinoma originating as a Marjolin's ulcer. *Dermatol Surg* 2004; **30**: 229-30.
13 Dubina M, Goldenberg G. Viral-associated nonmelanoma skin cancers: a review. *Am J Dermatopathol* 2009; **31**: 561-73.
14 Bastiaens MT, Hoefnagel JJ, Bruijn JA et al. Differences in age, site distribution, and sex between nodular and superficial basal cell carcinoma indicate different types of tumors. *J Invest Dermatol* 1998; **110**: 880-4.
15 Lo Muzio L. Nevoid basal cell carcinoma syndrome (Gorlin syndrome). *Orphanet J Rare Dis* 2008; **3**: 32.
16 Schwartz RA. The keratoacanthoma: a review. *J Surg Oncol* 1979; **12**: 305-17.
17 Schwartz RA. Keratoacanthoma: a clinico-pathologic enigma. *Dermatol Surg* 2004; **30**: 326-33; discussion 33.
18 Karaa A, Khachemoune A. Keratoacanthoma: a tumor in search of a classification. *Int J Dermatol* 2007; **46**: 671-8.
19 Stockfleth E, Ferrandiz C, Grob JJ et al. Development of a treatment algorithm for actinic keratoses: an European Consensus. *Eur J Dermatol* 2008; **18**: 651-9.
20 Cox NH. Guidelines for management of Bowen's disease: 2006 update. *Br J Dermatol* 2007; **156.**: 11-21.
21 Telfer NR. Guidelines for the management of basal cell carcinoma. *B J Dermatol* 2008; **159**: 35-48.

22 Enoch S, Miller DR, Price PE et al. Early diagnosis is vital in the management of squamous cell carcinomas. *Int Wound J* 2004; **1**: 165-75.
23 Krawtchenko N, Roewert-Huber J, Ulrich M et al. A randomised study of topical 5% imiquimod vs. topical 5-fluorouracil vs. cryosurgery in immunocompetent patients with actinic keratoses: a comparison of clinical and histological outcomes including 1-year follow-up. *Br J Dermatol* 2007; **157 Suppl 2**: 34-40.
24 Fariba I, Ali A, Hossein SA et al. Efficacy of 3% diclofenac gel for the treatment of actinic keratoses: a randomized, double-blind, placebo controlled study*Indian J Dermatol Venereol Leprol* 2006; **72**: 346-9.
25 Kjellgren KI, Ring L, Lindblad AK et al. To follow dermatological treatment regimens--patients' and providers' views*Acta Derm Venereol* 2004; **84**: 445-50.
26 Serup J, Lindblad AK, Maroti M et al. To follow or not to follow dermatological treatment--a review of the literature*Acta Derm Venereol* 2006; **86**: 193-7.
27 Bath-Hextall FJ, Perkins W, Bong J et al. Interventions for basal cell carcinoma of the skin. *Cochrane Database Syst Rev* 2007: CD003412.
28 van der Geer S, Ostertag JU, Krekels GA. Treatment of basal cell carcinomas in patients with nevoid basal cell carcinoma syndrome. *J Eur Acad Dermatol Venereol* 2009; **23**: 308-13.
29 Chan S, Dhadda AS, Swindell R. Single fraction radiotherapy for small superficial carcinoma of the skin. *Clin Oncol (R Coll Radiol)* 2007; **19**: 256-9.
30 Tappeiner H, Jesionek A. Therapeutische versuche mit fluoreszierenden stoffen. *Munch Med Wochenschr* 1903; **47**: 2042-4.
31 Dougherty TJ, Kaufman JE, Goldfarb A et al. Photoradiation therapy for the treatment of malignant tumors. *Cancer Res* 1978; **38**: 2628-35.
32 Kennedy JC, Pottier RH, Pross DC. Photodynamic Therapy with Endogenous Protoporphyrin IX. Basic Principles and Present Clinical Experience. *J Photochem Photobiol B* 1990; **6**: 143-8.
33 Moan J, Ma LW, Iani V. On the pharmacokinetics of topically applied 5-aminolevulinic acid and two of its esters. *Int J Cancer* 2001; **92**: 139-43.
34 Peng Q, Berg K, Moan J et al. 5-aminolevulinic acid-based photodynamic therapy: Principles and experimental research. *Photochem Photobiol* 1997; **65**: 235-51.
35 Ohgari Y, Nakayasu Y, Kitajima S et al. Mechanisms involved in delta-aminolevulinic acid (ALA)-induced photosensitivity of tumor cells: relation of ferrochelatase and uptake of ALA to the accumulation of protoporphyrin *Biochem Pharmacol* 2005; **71**: 42-9.
36 Hinnen P, de Rooij FW, van Velthuysen ML et al. Biochemical basis of 5-aminolaevulinic acid-induced protoporphyrin IX accumulation: a study in patients with (pre)malignant lesions of the oesophagus *Br J Cancer* 1998; **78**: 679-82.
37 Rodriguez L, Batlle A, Di Venosa G et al. Mechanisms of 5-aminolevulinic acid ester uptake in mammalian cells. *Br J Pharmacol* 2006; **147**: 825-33.
38 Vinciullo C, Elliott T, Francis D et al. Photodynamic therapy with topical methyl aminolaevulinate for 'difficult-to-treat' basal cell carcinoma. *Br J Dermatol* 2005; **152**: 765-72.
39 Rhodes LE, de Rie M, Enstrom Y et al. Photodynamic therapy using topical methyl aminolevulinate vs surgery for nodular basal cell carcinoma: results of a multicenter randomized prospective trial. *Arch Dermatol* 2004; **140**: 17-23.

40 Peng Q, Soler AM, Warloe T et al. Selective distribution of porphyrins in skin thick basal cell carcinoma after topical application of methyl 5-aminolevulinate. *J Photochem Photobiol B* 2001; **62**: 140-5.
41 Ahmadi S, McCarron PA, Donnelly RF et al. Evaluation of the penetration of 5-aminolevulinic acid through basal cell carcinoma: a pilot study. *Exp Dermatol* 2004; **13**: 445-51.
42 Wennberg AM, Larko O, Lonnroth P et al. Delta-aminolevulinic acid in superficial basal cell carcinomas and normal skin-a microdialysis and perfusion study. *Clin Exp Dermatol* 2000; **25**: 317-22.
43 van den Akker JT, Holroyd JA, Vernon DI et al. Comparative in vitro percutaneous penetration of 5-aminolevulinic acid and two of its esters through excised hairless mouse skin*Lasers Surg Med* 2003; **33**: 173-81.
44 Gerritsen MJ, Smits T, Kleinpenning MM et al. Pretreatment to enhance protoporphyrin IX accumulation in photodynamic therapy. *Dermatology* 2009; **218**: 193-202.
45 Smits T, Kleinpenning MM, Blokx WA et al. Fluorescence diagnosis in keratinocytic intraepidermal neoplasias. *J Am Acad Dermatol* 2007; **57**: 824-31.
46 Moseley H, Brancaleon L, Lesar AE et al. Does surface preparation alter ALA uptake in superficial non-melanoma skin cancer in vivo? *Photodermatol Photoimmunol Photomed* 2008; **24**: 72-5.
47 Moan J, Ma LW, Iani V. On the pharmacokinetics of topically applied 5-aminolevulinic acid and two of its esters*Int J Cancer* 2001; **92**: 139-43.
48 Gibson SL, Havens JJ, Foster TH et al. Time-dependent intracellular accumulation of delta-aminolevulinic acid, induction of porphyrin synthesis and subsequent phototoxicity. *Photochem Photobiol* 1997; **65**: 416-21.
49 Ericson MB, Sandberg C, Gudmundson F et al. Fluorescence contrast and threshold limit: implications for photodynamic diagnosis of basal cell carcinoma. *J. Photochem. Photobiol. B* 2003; **69**: 121-7.
50 Braathen LR, Paredes BE, Saksela O et al. Short incubation with methyl aminolevulinate for photodynamic therapy of actinic keratoses*J Eur Acad Dermatol Venereol* 2009; **23**: 550-5.
51 Sakamoto FH, Tannous Z, Doukas AG et al. Porphyrin distribution after topical aminolevulinic acid in a novel porcine model of sebaceous skin*Lasers Surg Med* 2009; **41**: 154-60.
52 Honeywell-Nguyen PL, Bouwstra JA. The in vitro transport of pergolide from surfactant-based elastic vesicles through human skin: a suggested mechanism of action. *J Control Release* 2003; **86**: 145-56.
53 Kaliszewski M, Kwasny M, Juzeniene A et al. Biological activity of 5-aminolevulinic acid and its methyl ester after storage under different conditions. *J Photochem Photobiol B* 2007; **87**: 67-72.
54 Juarranz A, Jaen P, Sanz-Rodriguez F et al. Photodynamic therapy of cancer. Basic principles and applications. *Clin Transl Oncol* 2008; **10**: 148-54.
55 Pispisa B, Venanzi M, Palleschi A et al. Intramolecular electronic energy transfer in peptides carrying naphthalene and protoporphyrin molecules: a spectroscopic and conformational statistics investigation*Biopolymers* 1995; **36**: 497-510.
56 Robinson DJ, de Bruijn HS, van der Veen N et al. Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic

therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect*Photochem Photobiol* 1998; **67**: 140-9.
57 Langmack K, Mehta R, Twyman P et al. Topical photodynamic therapy at low fluence rates--theory and practice. *J Photochem Photobiol B* 2001; **60**: 37-43.
58 Soler AM, Angell-Petersen E, Warloe T et al. Photodynamic therapy of superficial basal cell carcinoma with 5-aminolevulinic acid with dimethylsulfoxide and ethylendiaminetetraacetic acid: a comparison of two light sources. *Photochem Photobiol* 2000; **71**: 724-9.
59 Henderson BW, Busch TM, Snyder JW. Fluence rate as a modulator of PDT mechanisms. *Lasers Surg Med* 2006; **38**: 489-93.
60 Star WM, van't Veen AJ, Robinson DJ et al. Topical 5-aminolevulinic acid mediated photodynamic therapy of superficial basal cell carcinoma using two light fractions with a two-hour interval: long-term follow-up. *Acta Derm Venereol* 2006; **86**: 412-7.
61 de Haas ER, de Bruijn HS, Sterenborg HJ et al. Microscopic distribution of protoporphyrin (PpIX) fluorescence in superficial basal cell carcinoma during light-fractionated aminolaevulinic acid photodynamic therapy. *Acta Derm Venereol* 2008; **88**: 547-54.
62 Puizina-Ivic N, Zorc H, Vanjaka-Rogosic L et al. Fractionated illumination improves the outcome in the treatment of precancerous lesions with photodynamic therapy. *Coll Antropol* 2008; **32 Suppl 2**: 67-73.
63 Wiegell SR, Haedersdal M, Philipsen PA et al. Continuous activation of PpIX by daylight is as effective as and less painful than conventional photodynamic therapy for actinic keratoses; a randomized, controlled, single-blinded study. *Br J Dermatol* 2008; **158**: 740-6.
64 Wiegell SR, Haedersdal M, Eriksen P et al. Photodynamic therapy of actinic keratoses with 8% and 16% methyl aminolaevulinate and home-based daylight exposure: a double-blinded randomized clinical trial. *Br J Dermatol* 2009; **160**: 1308-14.
65 Alexiades-Armenakas M. Laser-mediated photodynamic therapy. *Clin Dermatol* 2006; **24**: 16-25.
66 Anderson RR, Parrish JA. The optics of human skin. *J Invest Dermatol* 1981; **77**: 13-9.
67 Attili SK, Lesar A, McNeill A et al. An open pilot study of ambulatory photodynamic therapy using a wearable low-irradiance organic light-emitting diode light source in the treatment of nonmelanoma skin cancer. *Br J Dermatol* 2009; **161**: 170-3.
68 Grapengiesser S, Ericson M, Gudmundsson F et al. Pain caused by photodynamic therapy of skin cancer. *Clin Exp Dermatol* 2002; **27**: 493-7.
69 Wiegell SR, Skiveren J, Philipsen PA et al. Pain during photodynamic therapy is associated with protoporphyrin IX fluorescence and fluence rate. *Br J Dermatol* 2008; **158**: 727-33.
70 Morton CA, Whitehurst C, Moore JV et al. Comparison of red and green light in the treatment of Bowen's disease by photodynamic therapy. *Br J Dermatol* 2000; **143**: 767-72.
71 Clark C, Bryden A, Dawe R et al. Topical 5-aminolaevulinic acid photodynamic therapy for cutaneous lesions: outcome and comparison of light sources. *Photodermatol Photoimmunol Photomed* 2003; **19**: 134-41.

72 Wiegell SR, Haedersdal M, Wulf HC. Cold water and pauses in illumination reduces pain during photodynamic therapy: a randomized clinical study. *Acta Derm Venereol* 2009; **89**: 145-9.
73 Skiveren J, Haedersdal M, Philipsen PA et al. Morphine gel 0.3% does not relieve pain during topical photodynamic therapy: a randomized, double-blind, placebo-controlled study. *Acta Derm Venereol* 2006; **86**: 409-11.
74 Holmes MV, Dawe RS, Ferguson J et al. A randomized, double-blind, placebo-controlled study of the efficacy of tetracaine gel (Ametop) for pain relief during topical photodynamic therapy. *Br J Dermatol* 2004; **150**: 337-40.
75 Langan SM, Collins P. Randomized, double-blind, placebo-controlled prospective study of the efficacy of topical anaesthesia with a eutetic mixture of lignocaine 2.5% and prilocaine 2.5% for topical 5-aminolaevulinic acid-photodynamic therapy for extensive scalp actinic keratoses. *Br J Dermatol* 2006; **154**: 146-9.
76 Wiegell SR, Wulf HC. Photodynamic therapy of acne vulgaris using 5-aminolevulinic acid versus methyl aminolevulinate. *J Am Acad Dermatol* 2006; **54**: 647-51.
77 Kasche A, Luderschmidt S, Ring J et al. Photodynamic therapy induces less pain in patients treated with methyl aminolevulinate compared to aminolevulinic acid. *J Drugs Dermatol* 2006; **5**: 353-6.
78 Moloney FJ, Collins P. Randomized, double-blind, prospective study to compare topical 5-aminolaevulinic acid methylester with topical 5-aminolaevulinic acid photodynamic therapy for extensive scalp actinic keratosis. *Br J Dermatol* 2007; **157**: 87-91.
79 Pagliaro J, Elliott T, Bulsara M et al. Cold air analgesia in photodynamic therapy of basal cell carcinomas and Bowen's disease: An effective addition to treatment: A pilot study. *Dermatol Surg* 2004; **30**: 63-6.
80 Halldin CB, Paoli J, Sandberg C et al. Transcutaneous electrical nerve stimulation for pain relief during photodynamic therapy of actinic keratoses. *Acta Derm Venereol* 2008; **88**: 311-3.
81 Borelli C, Herzinger T, Merk K et al. Effect of subcutaneous infiltration anesthesia on pain in photodynamic therapy: a controlled open pilot trial. *Dermatol Surg* 2007; **33**: 314-8.
82 Halldin CB, Paoli J, Sandberg C et al. Nerve blocks enable adequate pain relief during topical photodynamic therapy of field cancerization on the forehead and scalp. *Br J Dermatol* 2009; **160**: 795-800.
83 Paoli J, Halldin C, Ericson MB et al. Nerve blocks provide effective pain relief during topical photodynamic therapy for extensive facial actinic keratoses. *Clin Exp Dermatol* 2008; **33**: 559-64.
84 Moghissi K, Stringer MR, Dixon K. Fluorescence photodiagnosis in clinical practice. *Photodiagnosis Photodyn Ther* 2008; **5**: 235-7.
85 Brancaleon L, Durkin AJ, Tu JH et al. *In vivo* Fluorescence Spectroscopy of Nonmelanoma Skin Cancer. *Photochem Photobiol* 2001; **73**: 178-83.
86 Policard A. Études sur les aspects offert par des tumeurs expérimentales examinées a la lumière de Wood. *C R Biol* 1924; **91**.
87 Inaguma M, Hashimoto K. Porphyrin-like fluorescence in oral cancer: In vivo fluorescence spectral characterization of lesions by use of a near-ultraviolet excited autofluorescence diagnosis system and separation of fluorescent extracts by capillary electrophoresis. *Cancer* 1999; **86**: 2201-11.

88 Andersson-Engels S AnderssonS. Fluorescence imaging and point measurements of tissue: applications to the. *Photochem Photobiol* 1991; **53**: 807.
89 Straight RC, Benner RE, McClane RW et al. Application of charge-coupled device technology for measurement of laser light and fluorescence distribution in tumors for photodynamic therapy. *Photochem Photobiol* 1991; **53**: 787-96.
90 Hewett J, Nadeau V, Ferguson J et al. The application of a compact multispectral imaging system with integrated excitation source to in vivo monitoring of fluorescence during topical photodynamic therapy of superficial skin cancers. *Photochem Photobiol* 2001; **73**: 278-82.
91 Monici M. Cell and tissue autofluorescence research and diagnostic applications. *Biotechnol Annu Rev* 2005; **11**: 227-56.
92 Wagnieres GA, Star WM, Wilson BC. In vivo fluorescence spectroscopy and imaging for oncological applications. *Photochem Photobiol* 1998; **68**: 603-32.
93 Kollias N, Zonios G, Stamatas GN. Fluorescence spectroscopy of skin. *Vibrational Spectroscopy* 2002; **28**: 17-23.
94 Ericson MB, Berndtsson C, Stenquist B et al. Fluorescence demarcation of basal cell carcinoma cotrolled by histopathological mapping. In: *SPIE Medical Imaging*, Vol. 5370. San Diego, USA: SPIE, 2004: 1411-8.
95 de Bruijn HS, Meijers C, van der Ploeg-van den Heuvel A et al. Microscopic localisation of protoporphyrin IX in normal mouse skin after topical application of 5-aminolevulinic acid or methyl 5-aminolevulinate. *J Photochem Photobiol B* 2008; **92**: 91-7.
96 Stahl M, Bouw R, Jackson A et al. Human microdialysis. *Curr Pharm Biotechnol* 2002; **3**: 165-78.
97 Hocht C, Opezzo JA, Taira CA. Microdialysis in drug discovery. *Curr Drug Discov Technol* 2004; **1**: 269-85.
98 Herkenne C, Alberti I, Naik A et al. In vivo methods for the assessment of topical drug bioavailability. *Pharm Res* 2008; **25**: 87-103.
99 Kezic S. Methods for measuring in-vivo percutaneous absorption in humans. *Hum Exp Toxicol* 2008; **27**: 289-95.
100 Anderson C, Andersson T, Wardell K. Changes in skin circulation after insertion of a microdialysis probe visualized by laser Doppler perfusion imaging. *J Invest Dermatol* 1994; **102**: 807-11.
101 Clough GF. Microdialysis of large molecules. *The AAPS journal* 2005; **7**: E686.
102 Kreilgaard M. Assessment of cutaneous drug delivery using microdialysis. *Adv Drug Deliv Rev* 2002; **54 Suppl 1**: S99-121.
103 Krogstad AL, Jansson PA, Gisslen P et al. Microdialysis methodology for the measurement of dermal interstitial fluid in humans. *Br J Dermatol* 1996; **134**: 1005-12.
104 Holmang A, Mimura K, Bjorntorp P et al. Interstitial muscle insulin and glucose levels in normal and insulin-resistant Zucker rats. *Diabetes* 1997; **46**: 1799-804.
105 Braathen LR, Szeimies RM, Basset-Seguin N et al. Guidelines on the use of photodynamic therapy for nonmelanoma skin cancer: an international consensus. *J Am Acad Dermatol* 2007; **56**: 125-43.
106 Peng Q, Warloe T, Berg K et al. 5-Aminolevulinic acid-based photodynamic therapy. Clinical research and future challenges. *Cancer* 1997; **79**: 2282-308.
107 Morton CA, McKenna KE, Rhodes LE. Guidelines for topical photodynamic therapy: update. *Br J Dermatol* 2008; **159**: 1245-66.

108 Wallengren J, Hakanson R. Effects of capsaicin, bradykinin and prostaglandin E2 in the human skin. *Br J Dermatol* 1992; **126**: 111-7.
109 Nolano M, Simone DA, Wendelschafer-Crabb G et al. Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. *Pain* 1999; **81**: 135-45.
110 Surrenti T, De Angelis L, Di Cesare A et al. Efficacy of photodynamic therapy with methyl aminolevulinate in the treatment of superficial and nodular basal cell carcinoma: an open-label trial. *Eur J Dermatol* 2007; **17**: 412-5.
111 Horn M, Wolf P, Wulf HC et al. Topical methyl aminolaevulinate photodynamic therapy in patients with basal cell carcinoma prone to complications and poor cosmetic outcome with conventional treatment. *Br J Dermatol* 2003; **149**: 1242-9.
112 Haller JC, Cairnduff F, Slack G et al. Routine double treatments of superficial basal cell carcinomas using aminolaevulinic acid-based photodynamic therapy. *Br J Dermatol* 2000; **143**: 1270-5.
113 Bender J, Ericson MB, Merclin N et al. Lipid cubic phases for improved topical drug delivery in photodynamic therapy. *J Control Release* 2005; **106**: 350-60.
114 Bender J, Simonsson C, Smedh M et al. Lipid cubic phases in topical drug delivery: visualization of skin distribution using two-photon microscopy. *J Control Release* 2008; **129**: 163-9.
115 Smits T, Moor AC. New aspects in photodynamic therapy of actinic keratoses. *J Photochem Photobiol B* 2009; **96**: 159-69.