ALA-PDT; the treatment of non melanoma skin cancer re-illuminated

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ALA-PDT; the Treatment of Non-Melanoma Skin Cancer Re-illuminated

(ALA-PDT; de Behandeling van Non-Melanoma Huidkanker tweevoudig belicht)

Proefschrift

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"Quelle drôle de planète ! pensa-t-il alors. Elle est toute sèche, et toute pointue et toute salée. Et les hommes manquent d'imagination. Ils répètent ce qu' on leur dit……"

Le petit prince
Antoine de Saint-Exupéry, 1946.

Voor mijn geliefden :
Voor Frederik
Voor Cor en Corrie

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Introduction

Based on :
"Een nieuw licht op de dermatologie,
fotodynamische therapie in de praktijk"

Ellen RM de Haas

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General Introduction and Outline of the Thesis

Non melanoma skin cancer and its incidence, reason for concern

Non melanoma skin cancer (NMSC) is the most common cancer in Caucasian people (1,2). NMSC mainly consists of squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). Often Bowens disease (SCC in situ) and actinic keratosis are considered to be included although they are not invasive. It is an important and a growing healthcare problem. If current trends continue it is expected that there will be 80% more (new) skin cancer patients by 2015 compared to those in 2000. The number of BCC patients will increase by 78% and by 2015 there will be 26000 new cases of first/primary BCC compared to 15000 in 2000 in the Netherlands. The largest increase in incidence is expected to be in younger age groups 15-64 years (females 94%, males 66%), and due to an increase in superficial BCC (3,4). A typical BCC patient is female, becoming younger, and presenting with a superficial lesion. If current patterns of sun exposure do not change this increase in incidence will continue over time due to the ageing population (3,5). A serious rise in annual demand for care for non melanoma skin cancer will occur. Although NMSC is the most common cancer, death from NMSC is uncommon but it occurs. An estimated number of 1017 deaths due to squamous cell carcinoma (SCC) and 672 due to BCC in man and woman of all ages occurs yearly in Europe (6). In 20 % of the deaths due to skin cancer metastases of SCC are the cause of death(7).

The etiology of NMSC is endogenous and exogenous. Genetic predisposition like skintype (Fitzpatrick I-V) and/or genetic diseases (e.g. Basal Cell Nevus syndrome) are involved. The most important exogenous factor is exposure to ultraviolet light, sun exposure and tanning lamp exposure. Other riskfactors are exposure to carcinogens like tar, coal, arsenic, certain industrial oils, solvents, ionizing radiation (11,12). Medical conditions as human papilloma virus infections or immuno-suppression (e.g. in organ recipients)(13,14,15) or intense PUVA treatments (16) induce a risk for development of NMSC. Even tobacco smoking has been linked to the development of SCC(17).

The incidence on NMSC is hard to say because in most countries NMSC is not included in cancerregistry. In Germany the age-standardized rate for NMSC was 100.2 per 100 000 inhabitants for men and 76.6 per 100 000 for women per year (1998 and 2001), 80% of NMSC were BCC. It is estimated that more than a

million BCC's and SCC occur every year in the United States. In the Netherlands incidence rates for BCC have been calculated to be 92 for men and 79 for women per 100 000 person years (4). NMSC incidence is even generally recognized to be underestimated and is increasing (3,5).

The therapeutic options for NMSC include surgery (micrographic or conventional), topical chemotherapy (5-fluouracil), immuno-modulating therapy (topical imiquimod), cryotherapy, curettage & electrodessication, radiotherapy and photodynamic therapy. The optimal treatment is dependent on the patient and the characteristics of the tumour. In most SCC surgical excision is first choice treatment (20). In BCC the treatment of choice is highly dependent on the growing pattern of the BCC: morpheiform, nodular, or superficial. For morphemic BCC surgery is regarded to be the treatment of choice. For nodular and superficial BCC different modalities as surgery, cryotherapy, curettage & electrodessication or PDT are commonly used.(21,22, 23) For the non invasive Bowens disease surgery, topical chemotherapy , cryotherapy, and PDT are used in daily practice (24). Actinic keratosis is mostly treated with cryotherapy, although the other superficial targeting modalities are also possible options (25, 26).Just recently guidelines on the use of PDT for NMSC have been published (27) which indicate there certainly is place for it.

Phototherapy in Dermatology: Nothing new under the Sun

Light has fascinated man from the beginning of time and research into its fundamental properties and its effects on matter have been researched in fields as diverse as physics and astronomy, medicine and philosophy. Even before the birth of Christ light was used to treat the skin. The Egyptians used light in combination with chemicals to provoke healing effects. The therapeutic effects of light alone are well known and these properties were utilized for many centuries (28). In 1903 Niels Finsen received the Nobel price for medicine based on his use of phototherapy for the treatment of patients with lupus vulgaris and tuberculosis (29). Modern day photochemotherapy (PUVA), in which skin containing psoralens is exposed to long wave ultraviolet radiation (UVA) was introduced in 1976 (30).

Another type of photochemotherapy is photodynamic therapy (PDT). PDT involves the combination of a light sensitive molecule, the photosensitiser and light of an

appropriate wavelength. The photosensitiser mediates the transfer of light energy to tissue oxygen and results in the production of reactive oxygen species, notably singlet oxygen. Singlet oxygen leads to the destruction of critical cellular and tissue components. Singlet oxygen is responsible for the photodynamic effect.

History of photodynamic Therapy: Without Knowledge of History you won't understand the Present

PDT has a long history. At the beginning of the twentieth century Jesionek and Von Tappeiner used eosin and light to treat skin cancer (31). Von Tappeiner discovered oxygen to be essential in the process which he called photodynamic reaction (32). Hematoporphrin was the first studied photosensitiser by Hausmann (33) and many others. This has led to the development of HpD (Photofrin) a mixture of porphyrin-monomers, dimers and oligomers (34). Meyer Betz himself experienced skin photosensitivity after admission of hematoporphrin. A day after admission of hematoporphrin to himself, he went outside and got severe edema and erythema (35).Prolonged photosensitivity of the skin after HpD admission is a clinical problem, patients had to stay in the dark for a long time. This has led to investigations to develop other photosensitisers in search for the ideal one. An ideal sensitiser should be biological stable, photochemically efficient, selective to the target cells, and without toxicity to normal tissue. Second generation photosensitisers were introduced – porphyrin derivatives, chlorins, bacteriochlorins and porphyrin-like sensitisers, phtalocyanins (36, 37). A different approach was identified by Kennedy and Pottier in 1990 (38). Here a photosensitiser pre-cursor or pro-drug, aminolevulinic acid (ALA), which is part of the heme biosynthetic pathway, is administered exogenously. This leads to the accumulation of the endogenous photosensitiser protoporphyrin IX (PpIX). Since 1990 hundreds of research articles have been published on the mechanisms of action underlying ALA-PDT. Many have investigated its use for the treatment of non-melanoma skin cancer. A major advantage of ALA-PDT in dermatology is the topical use of the prodrug and the almost absence of prolonged skin photosensitivity. The body of evidence in the literature and the minimal side effects of ALA-PDT resulted in 2001 in the approval of ALA and blue light (Levulan®) for treating actinic keratosis in the United States by the FDA (Food and Drug Administration) (39-42). Some years later the esterderivative of ALA, methyl-

aminolevulinate (MAL, Metvix®) was approved in most European countries for the treatment of superficial basal cell carcinoma (sBCC) and Bowens disease (43-48). A historical overview of major experiments and publications which has been underlying the use of PDT or support the development of PDT especially ALA-PDT is shown in table 1. Until now the risks of ALA-PDT are limited. No mayor side effect has been reported but a contact allergy to MAL (49). No carcinogenic or other danger is recognized in using PDT (50,51) But we should not ignore lessons from history and remain alert and prepared to change concepts, thoughts and practice.

Table 1: History(52,53)

	3000 BC India	psoralens and light treatment used for vitiligo
	12th century ancient Egypt	psoralens and light used for leukoderma
	ancient Herodotus	famous greek physician, father of heliotherapy
Discovery Antarctica, Fabian von Bellinghausen	1820	
	1834	Kalbrunner:isolation of 5-methoxypsoralen from bergamot oil
	1871	Hausmann: first studies of biological effect hematoporphrin
First International Polar Year (IPY) Weyprecht:Solutions to the fundamental problems of meteorology and geophysics were most likely to be found near the Earth's poles.12 countries were involved, 15 expeditions to the poles sent out. Beyond the advances to science and geographical exploration, a principal legacy of the First IPY was setting a precedent for international science cooperation.	1882-83	
	1900	Raab: Paramecium caudatum died within 2 h after exposition to acridine orange and light, not after one of these
	1900	Prime: eosin orally used to treat epilepsy, failing treatment but dermatitis in sun exposed skin areas as result

Scott and Shackleton reached 82^{0}17'ZB, trying to reach the Pole	1902	
	1903	Niels Finsen: Nobel Prize for treatment tuberculosis with UV-light and development carbon arc phototherapy
	1904	Van Tappeiner & Jodblauer: photodynamic effect: description oxygen consuming reaction in protozoa
	1905	Von Tappeiner & Jesionek: experiences with eosine and artificial light treating skin cancer , lupus vulgaris and other dermatological conditions
Shackletons second try to reach to Pole, he reached 88^{0} 23'ZB	1907	
Roald Amudsen arrived at the south Pole	1911	Hausmann: hematoporphrin used as sensitiser in guinea pigs and mice
Scott arrived at the south Pole, but on the way back he died	1912	
	1913	Meyer-Betz: injected himself hematoporprin and became swollen after light exposition
World war I, la grande guerre	1914-18	
	1924	Policard: fluorescent porphrin localized in malignant tumour, sarcoma in rat when illuminated with Woods lamp
Second International Polar Year(IPY) 40 nations participated. it heralded advances in meteorology, magnetism, atmospheric science, and in the "mapping" of ionospheric phenomena that advanced radioscience and technology	1932-33	
World war II	1940-45	
	1942	Auler & Banzer: hematoporphrin more concentrated in certain tumours compared to surrounding tissue
	1948	Figge & Weiland: hematoporphrin selectively absorbed into other cells
	1951	Manganiello &Figge: studied effect of hematoporphrin in 3 head and neck cancer patients
	1955	Rasmussen& Taxdal: intravenously hematoporprin in various conditions including skin cancer studied

	1955	Peck: studied diagnostic properties of hematoporphrin in biliary surgery
	1955	Schwarz: demonstrated hematoporphrin to be mixture and developed hematoporphrin derivative (HpD)
The International Geophysical Year (GPY, also IPY),celebration of the 75th and 25th anniversaries of the First and Second IPYs was conceived by a number of post-WWII eminent physicists, including Sydney Chapman, James Van Allen, and Lloyd Berkner, at an informal gathering in Washington DC in 1950. These individuals realized the potential of the technology developed during WWII	1957-58	
Article 1 Arctic Treaty : Antarctica shall be used for peaceful purposes only	1959	
	1960th	Schwartz, Lipson, Baldes: studies in use HpD for tumour detection
	1961	Lipson: HpD used in bronchoscopy to localize suspected malignant disease
	1967	Gray: cervical and vaginal HpD fluorescent diagnostics (malignant & benign)
	1970th	PUVA used as treatment for psoriasis
	1971	Leonard & Beck: HpD tumour detection in head and neck cancer
	1972	Diamond: concept of tumour localizing fluorescence might be effective as treatment modality (Lancet)
	1975	Dougherty: hematoporphrin purified derivative successfully used in treatment cutaneous malignancies with red light
	1975	Kelly: human bladder cancer cell transplanted in mice can be destroyed by PDT
	1978	Dougherty: first large series skin tumours treated with PDT
	1983	Benson: demonstrated positive correlation between HpD fluorescence and histological severe dysplasia or carcinoma in situ.
	1984	Mc Caughan: esophageal cancer treated with PDT, in all good palliation

	1990	Kennedy: introduction topical sensitiser prodrug aminolevulinic acid (ALA)
	1990	Divaris: proposal chemical porphyria as novel means of photosensitization for PDT
	1992	Pottier: ALA metabolized to potent photosensitiser protoporphrin IX (PpIX)
	1998	Fritsch: ALA & MAL more concentrated in AK than adjacent skin but MAL more specific in keratotic cells
	2001	FDA /US approval Levulan ® kerastick (ALA&blue light) for AK
	2004	FDA/ US approval MAL for non hyperkeratotic AK in face and scalp.MAL (Metvix ®) registration in Netherlands for superficial BCC & Ak
	2006	MAL (Metvix ®) registration for M Bowen
fourth International polar year (IPY)covers two full annual cycles from March 2007 to March 2009 and will involve over 200 projects, with thousands of scientists from over 60 nations examining a wide range of physical, biological and social research topics. It is also an unprecedented opportunity to demonstrate, follow, and get involved with cutting edge science in real-time	2007-9	

Principles of ALA-PDT

In daily practice ALA-PDT is simple to perform; apply ALA wait for a defined time-interval typically 4 – 6 hours, and illuminate the lesion with visible light, delivering a defined lightdose. This simplicity belies the fact the mechanism(s) underlying PDT are complicated. ALA-PDT is a treatment modality that involves the conversion of ALA to PpIX in tumour cells. Absorption of light by PpIX initiates the transfer of energy to molecular oxygen present in the tissue. This results in the production of reactive oxygen species, notably singlet oxygen. Singlet oxygen is responsible for the destruction of tumour and normal cells within the treatment volume. There is clearly a number of important factors to consider. The combination of light, sensitiser and oxygen is influenced by application time, illumination time,

the response of surrounding tissue, vessels and cells, and cellular products such as cytokines. PDT damage is the sum of achieved effects. The sum of tissue responses to the presence of light sensitive drugs / molecules illuminated by the appropriate wavelength. Tissue and cellular responses lead to direct and indirect cell death and a physiological response.

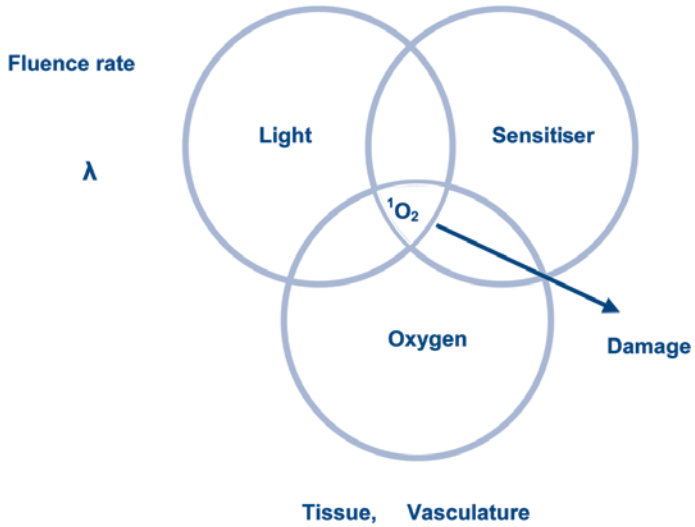

Figure 1: The range of interdependent parameters that determine the response of tissues to photodynamic therapy.

Porphyrin Synthesis in ALA-PDT

The administration of exogenous ALA to cells results in the accumulation of porphyrins, in particular PpIX. PpIX is an intermediate in the biosynthetic pathway of heme (figure 2), which consists of a number of steps that under normal conditions is tightly regulated by enzymatic control . The rate at which ALA is synthesized and enters the pathway is normally always less than the maximum rate of any of the subsequent steps. This means that intermediates in the pathway do not accumulate. However if the feedback mechanism between ALA and heme is bypassed by the presence of excess exogenous ALA, it is possible for one or more of the intermediates to accumulate. The extent to which this can happen is determined by the relationship between its rate of synthesis and the rate at which it is removed by the synthesis of

the next intermediate. The last step of the pathway in which iron is incorporated into PpIX to form heme, catalyzed by the enzyme ferrochelatase, is rate limiting and this leads to the accumulation of PpIX on the inner mitochondrial membrane. While PpIX has been shown to be the predominant photosensitiser accumulated in most cell types the addition of exogenous ALA can result in the accumulation of any of the sensitisers boxed in figure 2. While each of these has been shown to be photo-dynamically active in-vitro they are all highly soluble in water and are rapidly cleared from cells and tissues. In contrast PpIX is only slightly water soluble at physiological pH and shows a strong affinity for lipid membranes (38, 54)

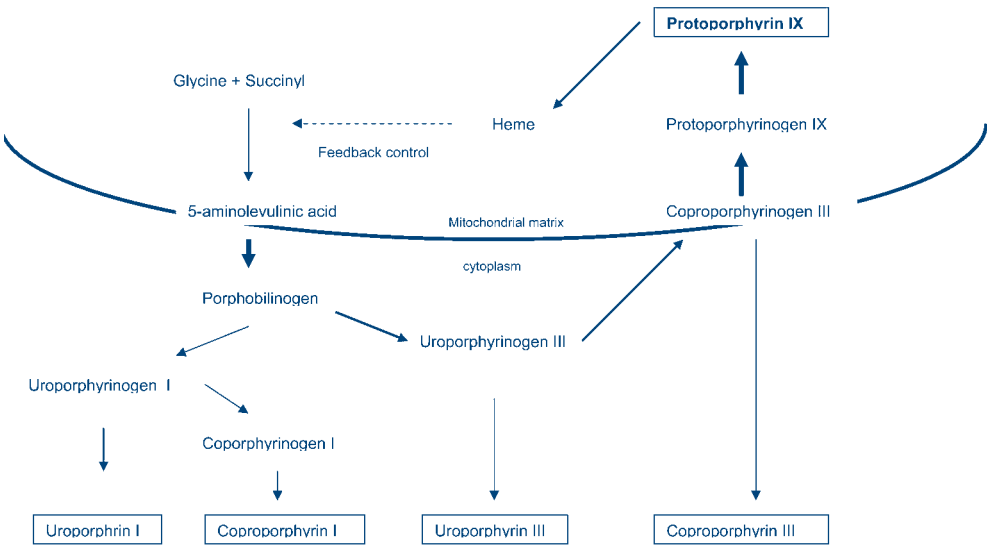

Figure 2: The bio-synthetic pathway of heme.
(adapted from In vivo analysis of the dynamics of photosensitiser fluorescence in photodynamic therapy, DJ Robinson University of Leeds UK Thesis)

Cellular uptake of the hydrophilic ALA is thought to be an active process.(55-57) cellular uptake takes time, in tumor cells the active uptake process is accelerated compared to normal cells (58). ALA esters, e.g.methylaminolevulinate are more lipophilic. As consequence of lipophilicity esters can also be taken up by a passive process, diffusion, through the epidermis (59, 60).ALA is known to be transported away from the area of topical application, MAL is accumulated intracellular and remains at the area of administration. Soon after cell penetration MAL is demethylated to ALA and therefore the subsequent metabolic steps of ALA and MAL

are thought to be identical. Although almost every cell is capable of PpIX production by the heme-bio-synthetic pathway, in malignant cells PpIX accumulation is ten fold higher than in normal counterpart cells (61). This has also been shown in tumour keratinocytes compared to healthy skin (62).

Distribution of PpIX in superficial Skin Cancer

Distribution of PpIX in cells, models and in vivo has been investigated extensively (59,63,64,65). At cellular level PpIX distribution has been found to be in mitochondria at first , but in other cellular components as well. The longer the incubation the more PpIX is distributed in different organelles such as endoplasmatic reticulum. Using topical ALA in a mouse model, normal skin and UV induced tumours, fluorescence was mainly located in tumour, in the epidermis and in hairfollicles. Four hours after topical application tumor selectivity was higher compared to fluorescence directly after ALA-application (66). In vivo PpIX has been shown in the superficial layers of nodular BCC using a topical application of 20% ALA. Penetration enhancers such as dimethylsulphoxide or longer application time resulted in deeper penetration of the ALA and more PpIX production (67).Penetration studies of ALA have shown penetration of at least 2 mm from the lesion surface into the depth.(68) Intracutaneous injected ALA in nodular BCC's has resulted in higher PpIX induced fluorescence as well (69). Intravenous injected ALA resulted in a more homogenous distribution of PpIX in the whole nodular ulcerative BCC (67). In superficial BCC PpIX fluorescence was shown in the full thickness of the tumor. A variable pattern of fluorescence was found inter and intra tumours.(70). Mostly in the above mentioned studies surface techniques were used to detect PpIX fluorescence in vivo.

Light

The penetration of light in tissue is obviously an important factor in the response of tissue to ALA-PDT. The penetration depth is influenced by the tissue optical properties and this will in turn have an impact on the light fluence (or dose) at depth and thus on the effect. These optical properties are characterized by absorption and scattering coefficients. The predominant absorbers in skin are oxy- and de-oxyhemoglobin,

melanin and water. Light scattering is also a very important factor that determines the distribution of light in skin. The absorption spectra of these components and the influence of light scattering mean that there is an 'optical window' between 600 and 670 nm where, long wavelength, red light penetrates tissue most efficiently. ALA-PDT is mostly performed using red light (610<λ<640nm) that overlaps with the absorption spectrum of PpIX. If light is assumed to be completely diffuse within tissue it is possible to show that the depth distribution of light has the formula

$$\Phi(z)= \Phi_0 k e^{\mu_{eff} z}.$$

The fluence rate, Φ, at depth z, is dependent on the fluence rate at surface, Φ_0, a constant factor (k) and an exponent that contains information about the absorbing and scattering properties of tissue. The factor k accounts for the effect of light scattering just below the surface of the tissue.(71,72,73).Measurements in human skin (non pigmented) have shown a mean μ_{eff} =0.359 mm^{-1} en k=2.63 at 630 nm. Therefore the effective penetration depth of red light in human skin is approximately 2.8mm (74). While there are likely to be clear differences in light penetration depth between tissues with different concentrations of absorbers, in particular blood, 2.8 mm represents a good estimate of the penetration depth of red light in skin. What is clear from this analysis is that ALA-PDT is not limited by the penetration depth of light and 2.8 mm is a sufficient penetration depth for most superficial non-melanoma skin cancer.

Photophysics and Photochemistry of ALA-PDT

Absorption of light by the photosensitiser results in the transfer of energy to electrons within the molecule as illustrated in figure 3. These electrons are able to share/ transfer energy in different ways within a molecule depending on their energetic status. Normally the photosensitiser is in a ground state (S_0) that is characterized by paired electrons. Absorption of a photon leads to excitation into S_1 S_2 S_3 etc. Energy transfer can then happen in two different ways: $S_1 \rightarrow S_0$ by emission of a photon (fluorescence) or $S_1 \rightarrow T_1 \rightarrow S_0$ by intersystem crossing to a triplet state of the photosensitiser. This triplet state of the photosensitiser is relatively long lived and this means that it can interact with its environment, in particular by transferring energy to surrounding molecules. This can happen in two different ways. Processes that are termed type I and II reactions. In a Type I reaction triplet state S reacts directly

with molecules of the substrate via electron transfer resulting in the production of oxygenated S and reduced S. In hypoxic environment reduced S can react with superoxide radicals producing superoxide ions ultimately leading to highly reactive hydroxyl radicals. In a Type II reaction energy is transferred to $^3O_2$ to produce singlet oxygen $^1O_2$ .Type II is an oxygen dependent reaction. Almost all of the evidence in the literature suggests that PpIX transfers energy to its environment via a type II reaction and singlet oxygen is the agent that is responsible for the damage to cells and tissues.(75,76)

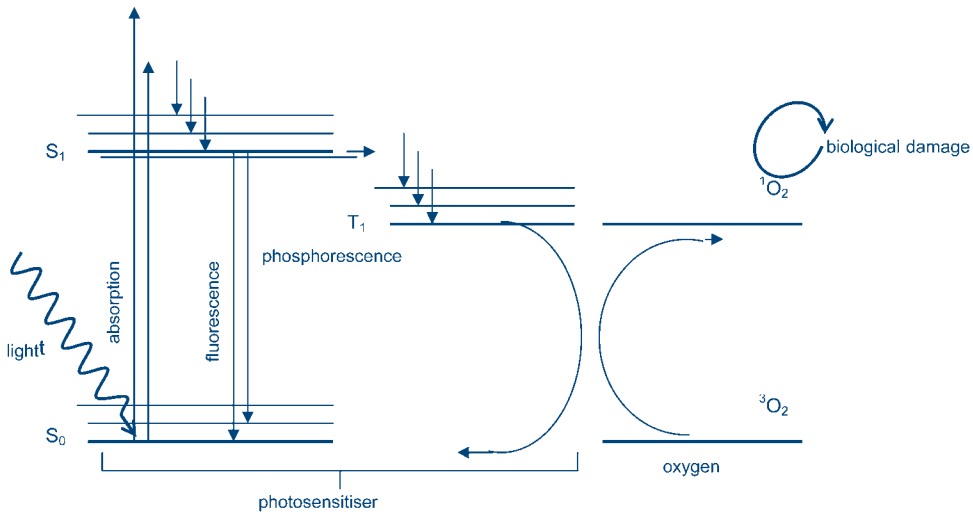

Figure 3: Photophysics; energy states involved in the transfer of energy from light to molecular oxygen to produce singlet oxygen adapted from DJ Robinson.

PHOTOBLEACHING

One important side effect of the generation of singlet oxygen by a photosensitiser is the process that is termed photobleaching. Photobleaching is the loss of absorption-capacity of the sensitiser, in effect the photosensitiser is being destroyed during the PDT process. Since photobleaching of PpIX is mediated by the same process that causes PDT damage it is a useful measure of the response of tissue to PDT and has been used as a predictor of tissue response (77). Following ALA-PDT, after the photobleaching of PpIX we and a number of investigators have observed continued

synthesis of PpIX and a reappearance of PpIX fluorescence. This is obviously an important factor when light fractionation is considered.

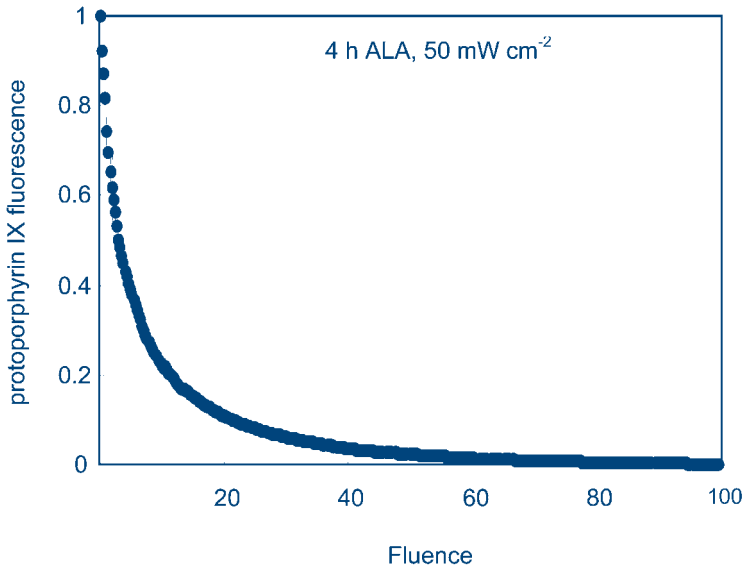

Figure 4: Photobleaching, from DJ Robinson (77)

Photobiology in ALA-PDT

As described above singlet oxygen is predominantly responsible for the response of tissues following ALA-PDT (78-80). The effect of a photosensitiser has been shown to be directly proportionate to the amount of singlet oxygen produced (81). Because of the very high reactivity and the short lifetime of singlet oxygen PDT induced damage is confined to the cell in which singlet oxygen is produced, which coincides with the localization of the photosensitiser.

Apoptosis, Necrosis and physiological effects of ALA-PDT

PDT leads to cell death by necrosis or by induction of apoptosis. Sublethal PDT damage still has an influence on cell biology and physiology and thus on tissue response. In general cell death is the result of two different pathways, necrosis and apoptosis. Apoptosis requires several active processes within the cell. It can be considered to be an ordered process of a badly damaged cell. An apoptotic cell disposes itself to be resolved completely. Necrosis is less well controlled and leads to

cell debris and crusts.

In general the process of apoptosis can be activated by two different pathways, the extrinsic and intrinsic pathway. The extrinsic pathway is initiated by binding of death ligands (e.g.FasL) to their cognate surface death receptors resulting in a signaling complex. Intracellular pro-caspase 8 and pro-caspase 10 are recruited and activated which in turn activates effector pro-caspases (3&7).

The intrinsic pathway is initiated by different extra- and intra-cellular stresses requiring permeabilization of the outer mitochondrial membrane which leads to the release of apoptogenic molecules (e.g.cytochrom-c) into the cytosol. Binding of these molecules leads to activation of pro-caspases (9) and downstream caspases (e.g.3). Apoptosis induced by PDT shows a rapid release of mitochondrial cytochrom-c-into the cytosol. In the photodynamic reaction, as soon as singlet oxygen is formed it will react with PpIX at the mitochondrial membrane to introduce apoptosis. Depending on the PDT-dose cells can respond to sub-lethal damage by engaging a rescue response or by cell death. Depending on the photodynamic stress either an apoptotic cell death or a necrotic cell death will occur. The balance between death and survival is regulated by signaling pathways (e.g. mitogen activated protein kinase cascades (MAPK)). These protein kinases are used to connect cell surface receptors to critical regulatory targets within the cell which control a divers array of cellular programs including cell death. PDT has been shown in cell lines, to be able to activate this regulating system (82).

Vascular effects

One of the responses to PDT is a destruction of tumour vasculature. Sensitive sites as vascular endothelium and the vascular basement membrane may get damaged leading to trombo-aggregation, release of vaso-active molecules, leucocyt-adhesion, increased vascular permeability, vessel constriction and haemorrhage(83,84). Systemic administered ALA has been shown to lead to accumulation of PpIX in vascular endothelial cells indicating vascular damage after PDT (85). In a mice model vascular damage has been shown also after topical ALA (86).

PDT influences on physiology

PDT can alter the function of different immune and non-immune cells leading to an inflammatory response(87,88). In models monocytes, dendritic cells, Langerhans cells and activated lymfocytes can be selectively sensitized by ALA.(89)

In Photophrin- PDT neutrophylic granulocytes play a very important role (90), in ALA-PDT a bystander role (91).

The mode of cell death is important "in general" but it is potentially especially important in light fractionation. A cell + vascular + tissue + immune response will already happen after the first illumination before another illumination will be performed. Fractionation may result in a changed "cumulative" response after the complete treatment.

Limitations of ALA and MAL-PDT and the Need for Optimization

The response of tissues to PDT that are photosensitised with PpIX following the application of porphyrin-precursors is limited by a number of factors. The penetration of ALA and MAL is limited by their diffusion through tissue layers, in particular the stratum corneum, but also by other tissue structures. ALA is known to be transported away from the area of topical application (92). This means that there is a limited amount of PpIX available for any single treatment. During illumination PpIX is also rapidly photobleached by the singlet oxygen generated during PDT. This limits the effective PDT dose that can be delivered in a single treatment as described in the following text. Despite the fact that ALA- and MAL-PDT is used clinically many research groups are investigating the mechanisms that underlie its effectiveness and are searching for methods to improve it.

Optimizing ALA-PDT; PpIX Re-Synthesis and Light Fractionation

In search to optimize the results of PDT different approaches have been investigated, both clinically and experimentally. Clinically, considering the minimal side effects of ALA-PDT repeating the treatment is an used option (93,94). In fact the recommended regime for MAL-PDT in the treatment of BCC and Bowens disease is two treatments, one week apart. Even further subsequent treatment sessions are often performed (95). Experimentally penetration enhancers have been used to enhance a single treatment. (96,97) Other techniques to enhance ALA penetration such as iontoforesis (67, 98) and tape stripping (99) have been investigated as well.

The effect to the distribution of PpIX of systemic ALA compared to topical has been investigated (67,100). However in patients only superficial necrosis was seen after systemic ALA-PDT for dysplasia in mouth and esophagus (101,102). A number of animal studies have demonstrated that the response to PDT after systemic ALA administration can be improved by modifying the illumination scheme, for example, by reducing the fluence rate to improve oxygenation (77, 103, 104). Another option is the use of light fractionation (105,106). Here the length of the dark interval is an important parameter. Short-term light fractionation (with one or more interruptions of seconds or minutes) may allow re-oxygenation during the dark interval that leads to more singlet oxygen deposition during therapy.(107) We have been carefully investigating the use of light fractionated ALA-PDT in different models using different fractionation schemes (108-111).In all models an increased effect was seen. In mice models more and deeper damage was seen, in a solid tumor model (transplanted rhabdomyosarcoma) a significant growth delay was seen after fractionated ALA-PDT. Our initial hypothesis was that the kinetics of PpIX re-synthesis was the underlying mechanism behind the increased response following light fractionation. However we have found the mechanism underlying the response to light fractionation is far more complex than we initially adapted. We have shown that the rate of PpIX re-synthesis after illumination is dependent on the PDT dose delivered (112). There is a fluence dependence for a fixed fluence rate; the higher the fluence the less PpIX is synthesized. And a fluence rate effect, where illumination at low fluence rate results in less re-synthesis for the same fluence. (Figure 5) Besides the fluence and fluence rate the application time and the length of the dark interval (113) have been shown to be important. In skin the dark interval should have a significant duration. Longer than 30 minutes is necessary to show an increased PDT effect by fractionation .Efficacy increases with the increasing length of the dark interval up to 2 hours (113). Mostly an application time of 4 hours was used in our experiments (like the most used clinical application time of ALA). In our animal work however a reduction in overall treatment time was achieved by using a shorter application time combined with an accordingly changed fluence for the different fractions of light. The concentration PpIX available at 2 hours is approximately 50% of that at 4 hours. It is therefore necessary to compensate for the reduced concentration of PpIX by increasing the fluence of the first illumination from 5 to 10 J cm^{-2} (112). The increase in efficacy using fractionated ALA-PDT is not due to an increasing PpIX content (113). There is an optimum of fractionation. In mice two fold ALA-PDT, in

which the illuminations are separated by a 2 hours dark interval, 5 + 95 Jcm^{-2} leads to significant more damage than 50 + 50 Jcm^{-2} which already leads to more damage than 100 Jcm^{-2} in a single illumination. (figure 6) The second fraction should be substantial larger than the first fraction to achieve a significant increase in PDT effect (113).

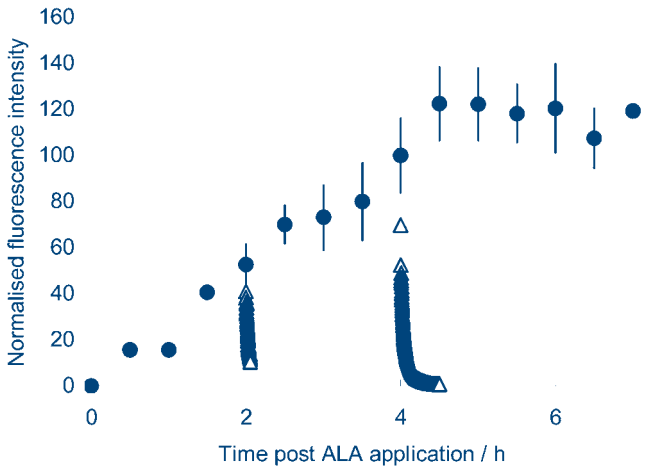

Figure 5: PpIX fluorescence kinetics 6h after ALA to normal mouse skin● and during two fold illumination scheme 10+90 J cm^{-2} with a two hours dark interval Δ from DJ Robinson (112)

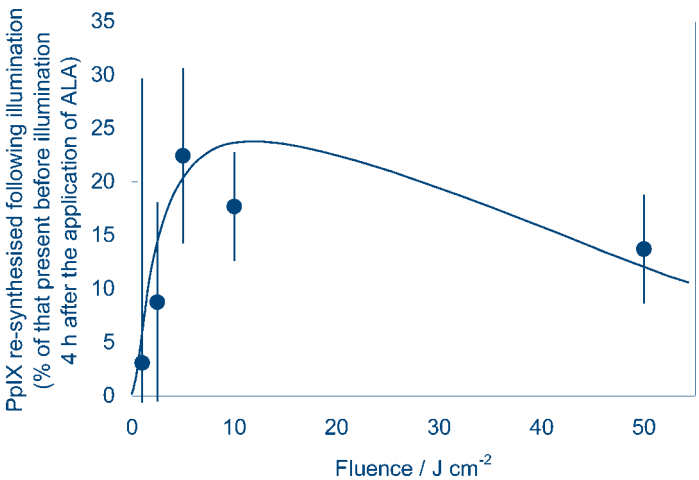

Figure 6: amount of PpIX that is synthesized during the 2 h dark interval between two lightfractions as a function of the fluence of the first light fraction 4 h after ALA application, DJ Robinson (112)

In conclusion, the results of our studies show that fractionated PDT results in significantly higher responses compared to PDT performed with a single illumination. The time interval between ALA application and the first light fraction and its relation to the synthesis of PpIX have been shown to be important. Also the influence of the fluence (rate) of the individual light fractions and the time interval between light fractions has been shown to be critical. Enhanced PDT response is achieved if two light fractions (a small and a substantially larger) are delivered 4 and 6 hours after a single application of ALA, separated by a 2 hours dark interval. These results have lead us to a first clinical study in sBCC using an illumination scheme 45Jcm^{-2} + 45Jcm^{-2} separated by a two hours dark interval. A follow up of mean 59 months (range 44-82) with a complete response rate of 84% and excellent cosmetic outcome in 88%.(114)

Aims and Outline of this Thesis

The purpose of this thesis was to investigate the role of light fractionated ALA-PDT in non-melanoma skin cancer. Our first aim was to show if the increase in effectiveness that has been demonstrated in a range of pre-clinical models and in a small pilot study using light fractionated ALA is also a significant effect in non-melanoma skin cancer. The second aim was to investigate the mechanism behind the use of light fractionation in clinical ALA-PDT. The third aim was to establish the importance of light fractionation in guidelines for treating NMSC with ALA-PDT.

The clinical studies will be described first; we designed a prospective randomized clinical study investigating the response of sBCC to fractionated ALA-PDT using a 4 hours application time after a single application of 20% ALA topically, a small first illumination of 20 J cm^{-2} followed by a dark interval of 2 hours and a second illumination of 80 J cm^{-2} compared to a single illumination scheme of 75 J cm^{-2} . The results will be described in chapter 2. A study investigating the same treatment scheme for Bowens disease will be described in chapter 3.

Investigating the mechanism of ALA-PDT we performed PpIX fluorescence studies related to immunohistochemical studies which are described in the following chapters. In chapter 4 PpIX fluorescence in human sBCC is described. In chapter 5 we describe the results we achieved investigating the mechanism of cell death especially apoptosis in human sBCC. In chapter 6 a comparison of ALA-PDT to MAL-

PDT in mice using fractionated illumination is described.

Evidence based medicine and guidelines are unmistakably very important in dermatology. In chapter 7 the results of an appraisal of the existing clinical guidelines in dermato-oncology are described. The results we achieved in Rotterdam for sBCC, nBCC, Bowens disease and AK which are described in chapter 8 should have consequences in future guideline development.

Dermatological oncological care is changing, not only because of the development of new treatments as PDT but also due to changed opinions of patients and their physicians. The impact of quality of life in treatment decision and outcome is the subject of chapter 9. In the general discussion we critically review our achieved results and place them in perspective to the future.

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Chapter 2

Fractionated illumination significantly improves the reponse of superficial basal cell carcinoma to ALA-PDT

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Abstract

Photodynamic therapy (PDT) of superficial basal cell carcinoma (sBCC) using topical 5-aminolevulinic acid (ALA) and a light fluence of 75–100 J cm^{-2} yields unsatisfactory long-term results. In several animal models, illumination with two light fractions 2 hours apart was considerably more effective than single illumination. Response is further enhanced if the fluence of the first light fraction is reduced, although the cumulative fluence is maintained. We compared the response of sBCC to a single illumination and 2-fold illumination scheme in which two light fractions of 20 and 80 J cm^{-2} are performed 4 and 6 hours after the application of a single dose of 20% ALA. We randomly assigned 154 patients with a total of 505 primary sBCC into two treatment groups. Two hundred and forty-three lesions were treated using a single illumination of 75 J cm^{-2} at a fluence rate of 50mWcm^{-2}. Fractionated PDT, at the same fluence rate, was performed on 262 lesions. The complete response (CR) following a 2-fold illumination scheme is significantly greater than that following a single light fraction ($P < 0.002$, log-rank test). Twelve months after therapy, CR rate to a 2-fold illumination is 97%, whereas the CR to a single illumination is 89%.

Introduction

Photodynamic therapy (PDT) of superficial (non-) malignant skin lesions, using topically applied 5-aminolevulinic acid (ALA) to endogenously generate the photosensitiser protoporphyrin IX (PpIX), was introduced by Kennedy et al. in 1990. Initially, high (80–100%) complete response (CR) rates were reported for topical ALA-mediated photodynamic therapy (ALA-PDT) in the treatment of superficial nonmelanoma skin cancer (1-5). However, comparing the results of ALA-PDT, a considerable variation in CR rates is observed; long-term response rates vary from 30 to 100% (6). To achieve higher CR, some investigators have treated lesions more than once (7,8). The success of topical ALA-PDT is dependent on several factors. In addition to penetration of ALA into the skin(-lesion) and the formation of a therapeutic concentration of PpIX, CR depends significantly on tumor thickness and duration of ALA application (9). Curettage before topical ALA-PDT may significantly improve CR rates, in particular for nodular basal cell carcinoma (10,11). In an attempt to increase the effectiveness of ALA-PDT, modified prodrugs of ALA have also been used such as a methyl aminolevulinate (MAL) and other esters (12-14). Although no comparative study has been performed to compare the clinical response using ALA and MAL, clinical studies published using MAL-PDT also show a variation in response rate. Horn et al. (15) reported a recurrence rate of 18% in a study of 94 basal cell carcinomas that were treated with MAL-PDT (two treatments a week apart, follow up (FU) 24 months. Soler et al. (16) reported the treatment of 350 curettaged nodular basal cell carcinoma and superficial basal cell carcinoma (sBCC) using MAL-PDT in two sessions with a CR of 79% (FU 22–24 months). The approved scheme of MAL-PDT in Europe consists of two treatments a week apart. Rhodes et al. (17) performed a comparative study using MAL-PDT versus surgery for nodular basal cell carcinomas, achieving 10% recurrence rate in the MAL-PDT group versus 2% recurrence rate in the surgery group. Recently, Van Iersel et al. (18) reported their results after primary surgery for a basal cell carcinoma being a 5-year cumulative risk of only 2.1%. These studies support the need to improve the response of sBCC to both MAL and ALA-PDT. We have performed series of pre-clinical studies investigating the effects of fractionating the illumination in PDT in a variety of model systems (19-24). We have shown increased efficacy of ALA-PDT using fractionated illumination with relatively long dark intervals between two light fractions (19). For topical administration, we have shown that a 2-hours dark interval is necessary to achieve a significant increase

in response. We have shown that choice of fluence (rate) for the first light fraction is critical, and that a high fluence for the second light fraction is necessary for maximal tissue response (23). Our first clinical pilot study using fractionated ALA-PDT using two equal light fractions of 45 J cm^{-2} with a 2-hours dark interval resulted in a CR rate of 84% (mean FU 59 months; range 44–82 months, n=67) (25). This was an encouraging result, but we were unable to show a statistically significant increase in response compared to a single illumination. This is probably owing to the choice of a non-optimized illumination scheme and the small number of lesions treated. Here, we report on a randomized comparative prospective open clinical study between a standard ALA-PDT treatment scheme using a fluence of 75 J cm^{-2}, 4 hours after the administration of ALA and a 2-fold illumination using 20+ 80 J cm^{-2}, delivered 4 and 6 hours after the administration of ALA. We also investigated the kinetics of PpIX fluorescence during the 2-fold illumination scheme and their relation to the mechanism of response.

Materials and Methods

Patients

All patients were diagnosed as having an sBCC within the Erasmus MC in Rotterdam. ALA-PDT was performed according to two treatment protocols, described in detail below, approved by the local ethics committee, according to the Declaration of Helsinki Principles. All patients gave informed consent. Diagnosis was determined histologically (4mm punch biopsy) and clinically in approximately equal proportions within each treatment group. Every patient had at least one histologically diagnosed primary sBCC. Our patient population consists of both first-line and secondary dermatological care. All patients are adult Caucasians. We treated 100 patients who had in total 243 lesions, using a single illumination scheme. Fifty-five patients with a total of 262 lesions were treated using a 2-fold illumination scheme, described below. Both groups were in the same age range; in the group that received a single fraction, the mean age was 57 years (minimum 32, maximum 81, median 57), and in the 2-fold illumination group, the mean age was 56 years (minimum 31, maximum 83, median 56). Also, both treatment groups included a similar number of higher risk patients as shown in Table A .

Table A: High risk patients.

	75 Jcm^{-2}		20+80 J cm^{-2}	
Patient group	patients	lesions	patients	lesions
Immune compromised	7	7	2	6
Previous Radiotherapy	8	33	5	20
Goltz Gorlin syndrome	5	46	4	17
High sun exposure	2	16	2	28
Topical arsenic use	-	-	1	9

Immune compromised: HIV positive, organ recipient or using immuno-suppressive drugs
High sun exposure: patients who have lived more than 15 years in tropical countries and had Fitzpatrick skin type 1.

ALA application and local anesthesia

A topical ALA ointment we used was prepared by our Hospital Pharmacy using 20% ALA (FLUKA, The Netherlands) in Instilagel (Medeco BV, Oud Beijerland, The Netherlands). Instilagel was used as vehicle because it contains lidocaine (2%), which is considered a possible advantage in pain management (26). The ointment was freshly prepared and stored in a refrigerator and used within 3 days. Before application of ALA, crusts and scaling were gently removed using a disposable curette. The lesion was covered with a margin of 1 cm and dressed with a semipermeable dressing (Tegaderm 3M, The Netherlands) and light-protecting covering (aluminium foil). Patients were instructed to stay out of the cold because of the negative effect of low temperatures on ALA metabolism (27). In addition to the topical anesthetic in the ALA ointment, patients received paracetamol, lidocaine (without epinephrine), or bupivacaine, if required.

Light sources and illumination scheme

Three light sources were used in this study. A 630nm diode laser (Carl Zeiss, Oberkochen, Germany) was used to provide 630nm illumination. Light was coupled into a 600 µm optical fiber and projected onto the lesion using a combination of lenses to assure a uniform fluence rate across the beam diameter. Two commercially available broadband light sources were also used. The first had a spectral output between 590 and 650nm (Medeikonos, Gothenburg, Sweden). The second was a light-emitting diode, light source with a spectral output centered on 633nm with a bandwidth of 20nm (Omnilux, Waldmann, Tiel, The Netherlands). All three light sources were used to illuminate lesions with a margin of at least 5mm at a constant

measured fluence rate of 50mWcm^{-2}. It was necessary to shield areas outside this margin for both broadband light sources. In the single illumination group, lesions were illuminated 4 hours after the application of ALA to a fluence of 75 J cm^{-2}. In the 2-fold illumination group, lesions received light fractions of 20 and 80 J cm^{-2}, 4 and 6 hours after the application of ALA. ALA was applied once. Again, both light fractions were delivered at a fluence rate of 50mWcm^{-2}. During the 2-h dark interval between light fractions, lesions were covered with light-protective bandage. Table B shows the number of lesions treated with each light sources in each illumination group.

Table B: CR of lesions treated with each light source in each illumination group

	75 Jcm^{-2}		20+80 Jcm^{-2}		
Light source	n	CR (%)	n	CR (%)	P-value
Diode laser (630nm)	160	91	42	98	0.236
LED Omnilux (633nm)	21	85	152	97	0.347
Medeikones (590-650nm)	62	89	68	97	0.093
total	243	89	262	97	0.002

Fluorescence measurements PpIX fluorescence measurements were acquired from a subgroup of 17 lesions in the 2-fold illumination group to investigate the kinetics of PpIX fluorescence during therapy. In this subgroup, PDT was performed using the diode laser. Data were acquired before ALA application, immediately before and after the first illumination and immediately before the second illumination. Measurements were performed with a custom applicator attached to a fluorescenceimaging camera (TRICAM, Storz, Tuttlingen, Germany). A uniform excitation light field (400–450 nm) was obtained from a ring of filtered blue light-emitting diodes mounted around the camera. Fluorescence emission from PpIX was collected over a period of 0.25 seconds. Scattered excitation light was blocked using a longpass filter (Schott KV 500). Fluorescence emission from PpIX (600–710 nm) was collected over a period of 0.25 seconds. Images acquired at different time points are registered under translation and rotation using landmarks in the skin surrounding the lesion (e.g. hair follicles). Typically, a rectangular area of 4-6 cm is imaged. Within this area, a circular region of interest approximately 0.25cm in diameter is defined and the average pixel intensity was used as a measure of the fluorescence intensity from an individual lesion. Measurements on a sheet of white plastic were used as

a reference to correct the fluorescence measurements of BCC for any changes in the output of the excitation light source or the sensitivity of the detection system. Autofluorescence was measured before each ALA application and was subtracted from the PpIX fluorescence intensity acquired before and after each illumination.

Response and Follow up

We defined CR as the absence of clinical visual basal cell carcinoma. All patients treated in our department are seen for FU according to the national dermatological guidelines of the Netherlands (NVDV). In the first year after treatment, FU was performed four times a year, thereafter twice yearly. Patients with a propensity of developing numerous skin lesions were seen more frequently in an individualized scheme. FU ends 5 years past the last treated basal cell carcinoma. Some of the patients are referred to us by peripheral primary dermatologists and are treated as secondary dermatological care patients and then referred back to their primary dermatologist for further FU. We only considered FU performed by our staff members and residents within our University hospital. A minimal FU of 12 months is necessary for inclusion in the study. In the single illumination group, FU varied from 12 to 41 months, mean 21 months. In the 2-fold illumination group, FU varied between 12 and 32 months, mean 17 months. Lesions that did not respond to therapy or recurred in the first 12 months after treatment were included in the data analysis as NR or recurrence. Cosmetic outcome was not the primary goal of the study, but remarks of patients and dermatologist were asked for and noted. The patient and dermatologist judged cosmetic outcome to be either good, fair, or poor.

Statistical analysis

Kaplan–Meier analysis was performed on relative CR rates after therapy and the log-rank test was used to compare the significance of differences between treatment groups. The primary and overall response rates of lesions treated with different illumination schemes were compared using Fisher's exact test. Differences in fluorescence intensity were compared using Student's t-test. $P<0.05$ was considered statistically significant.

Results

A 2-fold illumination scheme of 20+80 J cm^{-2} with a 2-hours dark interval results in a significantly better clinical response to ALA-PDT over a single light fraction The relative CR of lesions to ALA-PDT using a single light fraction and a 2-fold illumination scheme is shown in Figure 1.

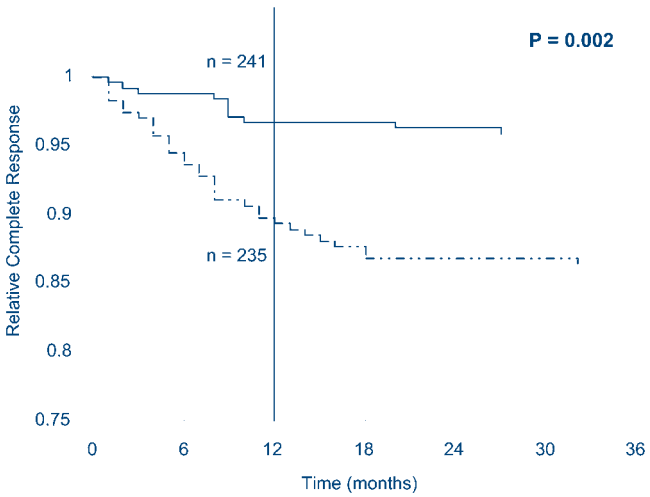

Figure 1: Kaplan-Meier curves for disease-free rates for relative complete response of superficial basal cell carcinoma using a single light fraction of 75 J cm^{-2}, 4 hours after the application of ALA (— -- — --) and a two-fold illumination scheme of 20 + 80 J cm-2 4 and 6 hours after the application of ALA. (———).

The CR using a 2-fold illumination is significantly greater than that following a single light fraction (P<0.002, log-rank test). Twelve months after therapy, the relative CR in the 2-fold illumination group is 97%, whereas the corresponding CR in the single illumination group is 89%. Of the 243 lesions in the single illumination group, 32 lesions failed to respond or recurred during the FU period. Of these lesions, 10 lesions were retreated with ALA-PDT, with five lesions receiving a second single illumination and five were retreated using a 2-fold illuminated scheme; all resulted in CR (minimum FU 12 months). These lesions were not included as responders in our statistical analysis of CR. The remaining 22 lesions were surgically excised and consisted 17 nodular, one micro-nodular, two morphemic, and two sBCC. Only 10 of the 262 lesions in the 2-fold illumination group did not show CR. Five lesions were excised and found to be nodular (n=3) or morphemic (n=2) basal cell

carcinomas and five were retreated with 2-fold illumination ALA-PDT and responded (FU 7, 8, 11, 14, and 16 months without recurrence). These were included as non-responders, although after a second treatment CR was seen. A sub-group analysis in which only histologically proven sBCCs are included shows a similar pattern of CR between the single- and 2-fold illumination groups. Twelve months after therapy, the relative CR in the 2-fold illumination group is 98%, whereas the corresponding CR in the single illumination group is 85% ($P<0.0003$, log-rank test). PDT was well tolerated in both illumination groups, and all patients completed therapy. A minority of patients (14%) required pain relief in addition to 2% lidocaine already present in the ALA ointment. The majority of patients, 133 out of 154 (86%), did not require additional pain relief during or after the therapeutic illumination. The details of patients who required pain medication are shown in Table 1 (see below).

A greater number of patients required pain relief in the 2-fold illumination group than in the single illumination group. In the single illumination group, five patients required pain relief for six of 32 treated lesions. In the 2-fold illumination group, 15 patients required pain relief for 44 of 64 treated lesions. There was no consistent difference in pain during each of the 2-fold light fractions, although in general patients reported more pain during the first illumination. Pain resolved quickly during the dark interval. Our pre-clinical animal data suggested that the acute response to therapy might be more severe in the 2-fold illumination group. In the 2-fold illumination, crusts formed following therapy in 15 lesions in six patients. In the single illumination group, crusts were seen in two lesions in two patients.

Table 1: painmanagement (cosmetic outcome in these lesions good)

Paracetamol	patient	single	lesions	Localization	Two fold	lesions	Localization	remarks
	A				x	4	trunk	4 out of 7 were painfull, all 6 in single not, pain during one treatment day, other days not, all lesions on trunk
	B	x	1	vertex				Taken after illumination
	C		4	face, trunk	x	2	face	Taken in dark interval
	D		5	arm	x	2	arm, face	Taken in dark interval
	E		2	trunk	x	7	arm 3, trunk 3, leg 1,	Taken in dark interval
	F		15	face 2 trunk 10 arm 3		19	trunk 13 arm 6	Not used but at end of day tired and little pain in two fold treated lesions
	G	x	1	face		1	face	
	H				x	2	face	Complaint afterwards, responded no pain at treatment time
	I				x	4	Leg	Taken in dark interval
	J				x	4	trunk 2 leg 2	Taken in dark interval (2) Taken in advance(2)
lidocain	K				x	1	face	Pre second fraction
	L				x	1	face	Pre second fraction, crusts+
	M				x	4	face 1 arm 2 trunk 1	Pre second fraction, rheumatic patient
	N	x	1	arm				
	O				x	2	trunk	Pre second illumination
	P	x	2	face				During illumination
bupivacain	Q				x	1	vertex	Block, pre first fraction
	R				x	4	face	Block pre first fraction and local during first fraction
	S	x	1				face	During illumination
	T				x	1	vertex	Block pre first fraction, still painfull in second fraction
	U				x	2	arm	During first illumination
total	21	5	32		15	61		

Despite crust formation, cosmetic outcome was good in all lesions. The relationship between acute response and the need for pain relief is shown in Table 1. One patient showed a pustular skin reaction in 11 of 16 lesions, which lasted 5 days. This was cultured and proven to be nonbacterial. A small number (19) showed persistent hypopigmentation at the illumination site 1 year after therapy. There was no significant difference in response rates for the different light sources used. The response rate following a 2-fold illumination scheme was greater for each light source. We found no statistically significant difference between the response rates for the different light sources within each illumination group. A comparison of the response of lesions to a single- and a 2-fold illumination scheme for each individual light source is shown

in Table B (see above in materials and methods).The kinetics of PpIX fluorescence were in accordance with that found in pre-clinical animal models. Figure 2 shows the mean fluorescence intensity present before and after the first light fraction of 20 J cm^{-2} and 2 hours later, immediately before the second light fraction.

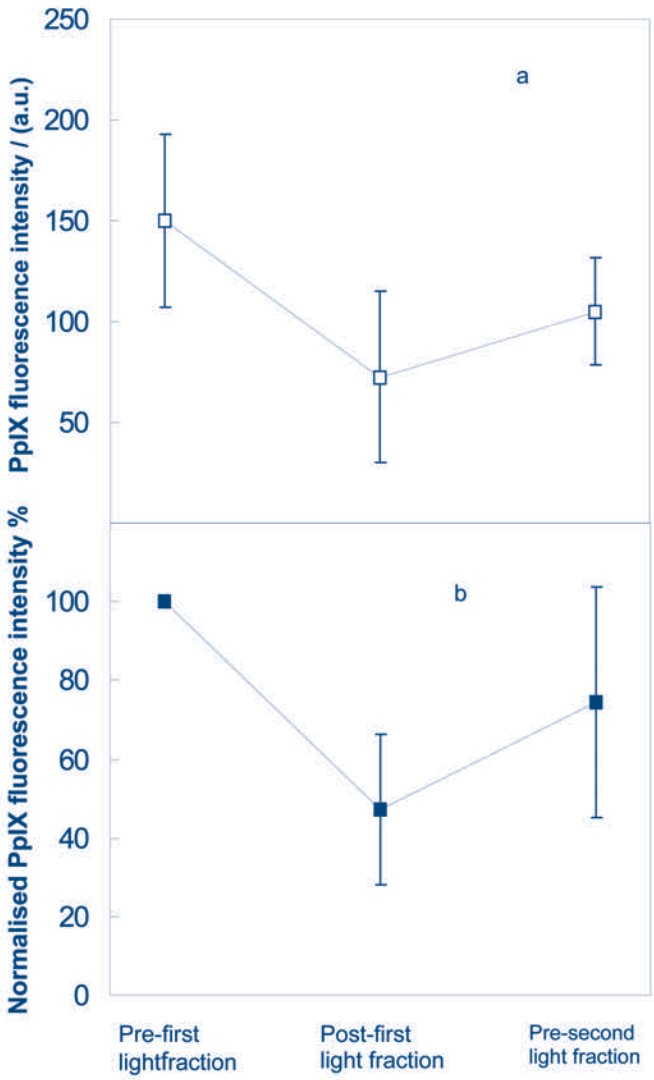

Figure 2: The kinetics of PpIX fluorescence intensity in sBCC during ALA-PDT with a two-fold illumination scheme (a). The relative PpIX photobleaching during therapy with 20 J cm^{-2} and the re-synthesis during the dark interval between light fractions (n =17 lesions), (b)

The PpIX fluorescence before the first light fraction varied considerably, between the lesions of each patient as well as between patients. When the fluorescence of all lesions is normalized to 100%, the mean fluorescence after the illumination is 47% and rises to 69% before the second illumination. There was considerable variation in PpIX fluorescence between lesions and between patients. Likewise, photobleaching varied widely, despite fixed illumination parameters. The average extent of photobleaching of 47% during the first light fraction is similar to that we have found in our pre-clinical studies. The amount of re-synthesis during the dark interval and the total amount of PpIX utilized in a single illumination compared to that in a 2-fold illumination scheme is also consistent to that we have seen in our pre-clinical studies (23).

Discussion

We have demonstrated that a statistically significant improvement in CR rate can be achieved using a light fractionation scheme with a 2-hours dark interval between light fractions of 20 and 80 J cm^{-2} compared to a single light fraction (97% versus 89% CR, 12 months FU, $P<0.002$, log-rank test). The lower response rate found with a single light fraction (89%) is similar to our previous results (4) and is consistent with the other investigators who have performed ALA-PDT in a single treatment session. Our choice of 2-fold illumination scheme is based on data from a range of pre-clinical animal studies that showed that following light illumination, tissues continue to synthesize PpIX. Apparently, the heme synthesis cycle is still (partly) intact and there is still ALA available to be converted into PpIX. PpIX fluorescence has been observed after PDT with both systemic ALA and topical ALA (19-22,28,29). However, these pre-clinical studies also demonstrate the complexity of the response of tissues to ALA-PDT and highlight the challenge for optimization (23). It is not simply a matter of performing a second illumination at some arbitrary time point after a standard single illumination. The time interval between light fractions is a very important consideration. A 2-hour time interval is necessary to yield a significant increase in response. Also, the fluence delivered in the first light fraction has a dramatic impact on the response to a 2-fold illumination. Although the response to a light fractionation scheme of 50+50 J cm^{-2} with a 2-hours dark interval is greater than a single illumination of 100 J cm^{-2}, the increase is not statistically significant.

Reducing the fluence of the first light fraction to 5 Jcm^{-2} and delivering a large fluence in the second (95 J cm^{-2}) significantly increases the effectiveness of PDT. In fact, this represents the most effective illumination scheme that we have yet devised. A similar pattern of response to light fractionation seems evident in clinical ALA-PDT of sBCC. In a clinical pilot study treating sBCC with two equal light fractions of 45+45 J cm^{-2}, separated by a 2-hours dark interval, we have shown an increase in long-term CR over that reported by other investigators (25). We were, however, not able to show a statistically significant improvement in CR at 12 months over a single illumination of 75 J cm^{-2}. This study shows that reducing the fluence of the first light fraction to 20 J cm^{-2} and maintaining that of the second light fraction to deliver a cumulative fluence of 100 J cm^{-2} results in a significant improvement in CR rate, just as predicted by our pre-clinical animal studies. We note that one other factor influenced the design of the clinical illumination scheme. sBCCs are in general more pigmented and significantly thicker than normal mouse epidermis. A higher light fluence of 20 J cm^{-2} was used, instead of the 5 J cm^{-2} that found to be optimal in our animal study, to ensure the deposition of sufficient fluence at the base of each lesion. The cumulative fluence in each of the illumination groups is not equal. A fluence of 75 J cm^{-2} was delivered in a single light fraction compared to 100 J cm^{-2} in the 2-fold illumination scheme. This is a direct consequence of our intention to deliver a large fluence in the second light fraction (23). The influence of this additional cumulative fluence on the clinical response we observe is an important issue. A number of pre-clinical studies have shown that photobleaching of the PpIX limits the PDT dose that can be delivered in a single light fraction at fixed fluence rate (30). We have shown in normal mouse skin that 100 J cm^{-2} does not result in significantly more damage than 50 J cm^{-2}. The relationship between response to ALA-PDT and fluence has not been systematically investigated in the clinic. Only Oseroff (31) has emphasized the importance of light fluence in the treatment of sBCC by topical ALA-PDT, and this regards the delivery of very high light fluences at high fluence rate. He reported that a CR rate of 95% after 200 J cm^{-2} (at a fluence rate of 150mWcm^{-2}) fell to 70% after 150 J cm^{-2}. Most other investigators have applied both lower light fluence (rate) and cumulative fluences both below 75 J cm^{-2} and above 100 J cm^{-2}, with little evidence for a correlation between fluence and response. Large variations occur in our fluorescence data. Several factors may be involved here, such as variations in tissue optical properties, in oxygenation, and in heterogenity of the PpIX distribution in the lesions (32). The variations in ALA-

induced sBCC fluorescence reported by af Klinteberg et al. (29) seem somewhat less, but these authors only report standard errors. Golub et al. (33) also measured large variations in ALA-induced fluorescence on normal human skin and psoriasis and actinic keratosis lesions. Such variability is widely observed in ALA-induced fluorescence of normal human skin and skin lesions (25,34,35,36). Accepting the large variations that seem to be a feature of clinical fluorescence measurements, we do see a similar trend in the kinetics of PpIX fluorescence after illumination with 20 J cm^{-2} . Importantly, we note that the original rationale for performing a 2-fold illumination scheme was to utilize additional PpIX that continued to be synthesized after PDT with a single light fraction. Our recent clinical data (25) and pre-clinical data (23) do not support the hypothesis that significantly more PpIX is utilized in the 2-fold illumination scheme, as 50+50 and 5+95 J cm^{-2} result in approximately the same amount of cumulative PpIX photobleaching ($P<0.12$, Student's t-test). The spectral output of the light sources used in this study may have influenced the response to a 2-fold illumination scheme. Our pre-clinical studies have shown that the response of tissues to PDT using the 2-fold illumination scheme is quite sensitive to the small differences in fluence and fluence rate of the first light fraction. Some investigators have suggested that there may be differences in response to PDT with (a) light sources that deliver a lower effective fluence rate by virtue of the overlap of their spectral output with the absorption spectrum of PpIX (37) and (b) light sources that additionally excite the fluorescent photoproducts of PpIX (38). The fact that we did not see differences in response between light sources suggests that these effects are small and do not impact significantly on the effective dose of the first light fraction. This means that the current fluence and dark interval findings in the current study are also applicable for the large proportion of investigators that use commercially available non-laser light sources. Our data stratified by light source (Table B) show that there is approximately the same increase in response of all the light sources investigated. We expect that statistical significance would be achieved if larger numbers of lesions were investigated. During the whole FU period, a significantly greater number of recurrent or non-responding lesions was observed in the single illumination group; 32/243 (13%) compared to the 2-fold illumination group 10/262 (4%) ($P<0.0002$, Fisher's exact test). Of the 32 lesions that did not show CR in the single illumination group, 20 were found not to be sBCC: nodular (18); micro-nodular (1); and morphemic (2) at recurrence. Of the 10 lesions that did not show CR in the 2-fold illumination group, five were found not to be sBCC at

recurrence, but nodular (3) and morphemic (2). The presence of nodular lesions in these data was not unexpected as diagnosis was based on clinical appearance for a significant proportion of lesions. Fifty-four percent and 49% of lesions were diagnosed clinically in the single- and 2-fold illumination group, respectively. Clinical diagnostic accuracy in a dermatology university faculty has been shown to be approximately 70% for BCC (39). If a similar accuracy were assumed in this study, we would expect to see 38 and 35 nodular lesions in our single- and 2-fold illumination groups, respectively. Three recurrent or non-responding nodular BCC's in the 2-fold illumination group represents a significantly lower number than expected ($P<0.0003$, Fisher's exact test). Although it was not out primary intention to treat nodular lesions, we have previously shown that the 2-fold illumination leads to deeper histological damage in pre-clinical models (21,23). Determining the maximum depth of BCC that can be treated with ALA-PDT is clearly an area for future study. The increased severity of the acute response following therapy shows a similar pattern to the increase in damage induced in our pre-clinical animal studies. This and the fact that we see an accompanying increase in CR after 12 months FU provides encouraging evidence for the value of PDT optimization in pre-clinical animal studies. In our animal studies, the increase in the effectiveness of the 2-fold illumination scheme leads to scarring in some cases. Although the clinical acute response is greater than a single illumination, the cosmetic outcome remains good in 90% of the lesions. We are also encouraged by the fact that patients did not experience significantly more pain during the 2-fold illumination scheme. A central aim of the current study was to achieve an optimal response to PDT in a single treatment session (i.e. with a single application of ALA). We note that repeating such an optimalized treatment regimen on subsequent treatment days may lead to an even higher CR rate in sBCC and/or a higher CR rate for other skin lesions. In conclusion, we have demonstrated a significant increase in the CR rate of sBCC to ALA-PDT using an illumination scheme in which two light fractions of 20 and 80 J cm^{-2} are delivered 4 and 6 hours after the application of ALA compared to a single illumination scheme of 75 J cm^{-2}. Further FU is now necessary to determine if this high CR rate is maintained. We note that the value of these results may extend beyond the skin, into other organs such as the esophagus and brain, where ALA-PDT is under investigation as a treatment modality (40,41).

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Chapter 3

Based on:

Response of Bowen's Disease to ALA-PDT using a single and a 2-fold illumination scheme

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Abstract

Objectives: To determine the response of Bowens disease (squamous cell carcinoma in-situ) to topical PDT using aminolevulinic acid using a single and a two-fold illumination.

Design: An open randomised pilot study.

Setting: A University Hospital in the Netherlands.
Patients and Patches: Forty patients were enrolled in this study with a total of 50 patches of Bowens disease. Twenty-five patches were randomly assigned to each illumination scheme group.

Interventions: Aminolevulinic acid 20% in Instilagel® was applied topically for a period of 4 hours. One group was illuminated with a single light fraction of 75 J cm^{-2}. The second group was illuminated with a two fold illumination scheme consisting of 20 J cm^{-2} and 80 J cm^{-2} separated by a 2 hours dark interval. The fluence rate for each light fraction was 50 mW cm^{-2}.

Main outcome measures: Complete response after a minimum follow up of 12 months. Healing time, adverse events and cosmetic outcome were determined as secondary outcome measures.

Results: Complete response rates to a single and two-fold illumination scheme, 12 months after therapy, were found to be 80% and 88% respectively. The combined average complete response rate in all patches was 84% after 12 months and 80% for a mean and median follow up of 24 months. Healing time in PDT treated areas was less than three weeks. Cosmetic outcome was good to excellent in all but 2 patches of Bowens disease, which were treated with two-fold illumination, due to persistent hyperpigmentation at 12 months.

Conclusion: Bowens disease can be successfully treated using ALA-PDT in a single treatment session and we found an increased response of Bowen's patches to PDT using a two-fold illumination scheme. This indicates the potential for a larger, higher powered study to determine if this effect is statistically significant.

Introduction

The use of 5-aminolevulinic acid photodynamic therapy (ALA-PDT) for the treatment of Bowens disease (squamous cell carcinoma in-situ) is gaining acceptance (1). However there is rather limited evidence for the most appropriate treatment regime and clinical follow up remains short. The treatment of first choice for Bowens disease is surgery. Five year response rates were recently published by the Australian skin cancer foundation and showed a recurrence rate of 19.0% after conventional surgery and 6.3 % after Mohs' micrographic surgery (2). In a comparative study investigating the response of Bowen's to ALA-PDT or cryotherapy, results were significantly in favour of PDT (3). In a comparative study of ALA-PDT versus topical 5-fluouracil recurrence rates at 12 months were 7% following PDT compared to 27% for 5-fluouracil (4). In a recent review of the literature Morton found 11 PDT studies using broadly similar protocols in which a response rate was found to be between 90 and 100% after 1 to 3 treatment sessions (5). An average recurrence rate of 12% was calculated from these studies. Importantly however the follow up period for these studies varied widely, between 3 and 36 months. Also in many cases therapy was repeated. Over the past decade we have been investigating the role of light fractionation in the response of tissues to ALA-PDT and in particular the enhanced response that can be achieved using ALA-PDT with long dark intervals. Several animal studies (6-9) have shown that a significant increase in response can be achieved with a small and large fraction separated by a dark interval of the order of 1 – 2 h between light fractions. While the specific light treatment parameters of each fraction are important for the optimisation of response (10) we have shown in a clinical pilot study that light fractionation increases the long-term response of superficial basal cell carcinoma (sBCC) to ALA-PDT (11). Most recently in a randomised comparative study of the response of sBCC to ALA-PDT we have shown a significantly higher complete response rate at one year (P=0.002, n=505) using a two fold illumination scheme (20 + 80 J cm^{-2}) with a two hours dark interval compared to a single illumination (12). In the light of these pre-clinical and clinical studies we have investigated the response of Bowens disease to ALA-PDT and the effect of fractionated illumination (20 + 80 J cm^{-2}) using a two-hours dark interval compared to a single illumination.

Methods

Study design

Fifty patches of Bowens disease were identified and randomly assigned into two equal treatment groups that recieved a single or two-fold illumination as described below.

Patients

The ethics committee of the Erasmus University Medical Centre granted approval for the study. Forty patients were recruited sequentially, on the basis of written informed consent, from those attending the department. The group consisted of 17 males and 23 females, 4 patients were organ recipients (1 liver, 1 heart, 2 kidney). The youngest was 49 years old, the oldest 91 years, mean age 74 years.

Bowen's patches

All patches were biopsy proven Bowens disease. Diagnosis was determined independently both by a pathologist and a dermatologist. The locations of the Bowen's patches are shown in table 1. The diameter of the patches ranged from 5 to 40 mm (mean 14.5 mm) and was more than 10 mm in 34 lesions and less than 10 mm in 16 lesions. The mean diameter of the patches in each illumination group was 13.4 and 15.6 mm respectively.

Table 1: Locations of Bowen's patches

	Number of lesions	75 J cm^{-2}	20 + 80 J cm^{-2}
Hand	8	3	5
Arm	1		1
Face			
Check / nose	3	1	2
Eyelid	2	1	1
Ear	7	5	2
Frontal/temporal	1		1
Scalp	1	1	
Upper leg	4	1	3
Lower leg	11	5	6
Trunk	12	8	4
Total	50	25	25

ALA application and lesion preparation

Topical ALA ointment was prepared by our University Pharmacy using 20 % aminolevulinic acid (FLUKA, The Netherlands) in Instilagel® (Medeco B.V. The Netherlands). The ointment was freshly prepared and stored in a refrigerator and used within 7 days. Instilagel® contains 20.9mg/ml of Lidocaine hydrochloride, and hydroxyethylcellulose water and a number of preservatives. This ointment was used in our earlier work and has been shown to be stable by our Pharmacy. Surface scale or crusts were gently removed. ALA ointment was applied for a period of 4 hours, with a margin of 1 cm around each patch. Patches were covered with a semi-permeable dressing (Tegaderm®, 3M) and a light protecting Aluminium foil between ALA administration and therapy.

Anaesthesia

We administered local anaesthesia (Lidocaine 2% without epinephrine) using a field-block technique only if patients required it.

Illumination schemes

The fractionated illumination scheme used in the present pilot study is based on an optimised scheme determined in pre-clinical models (6-10) and validated for the treatment of sBCC (11,12). Four hours after the application of ALA patches in the single illumination group were illuminated with a fluence of 75 J cm^{-2}, at a fluence rate of 50 mW cm^{-2}. In the two-fold illumination group patches were illuminated 4 and 6 hours after the application of ALA with fluences of 20 J cm^{-2} and 80 J cm^{-2} separated by a dark interval of 2 hours. Light in each fraction was delivered at a fluence rate of 50 $mWcm^{-2}$ ALA was administered once. In the dark interval patches were covered with a semi-permeable and light protective dressing (Tegaderm® and Aluminium foil).

Light sources

We have previously shown in sBCC that the light source does not significantly effect the response to ALA-PDT using light fractionation (12). Therefore two light sources were used in this study. A diode laser (Carl Zeiss, Oberkochen, Germany) was used to provide 630nm illumination and an LED light source (Omnilux®, Waldmann, Tiel, The Netherlands) with a spectral output centred on 633 nm with a bandwidth of 20 nm. Light from the laser was coupled into a 600 μm optical fibre and projected

onto the patch using a combination of lenses to assure a uniform fluence rate across the beam diameter. Patches were illuminated with a 5 mm margin. The diode laser was used to illuminate 16 patches the remaining 34 used the LED light source. A fan and/or wet gauze was used to cool the area of skin surrounding the lesion during the illumination.

Follow up and clearance rate

Patients were reviewed initially after 4 weeks and then at 3 monthly intervals. Response was classified as complete response being no clinical evidence of disease, with macroscopically normal skin at the treated site. Patches that responded partially were considered to be non-responders.

Adverse events, healing time and cosmetic outcome

No serious adverse effects were seen. All patients suffered a degree of discomfort during treatment but all finished the therapy. At first follow up patients were asked the time it took for the patches to heal. Healing was also assessed at the first follow up visit. Cosmetic outcome was scored good, fair or poor both by the dermatologist as the patient at 12 months.

Statistical Analysis

The primary response and recurrence rates of patients treated with different illumination schemes were compared using Fisher exact test. Kaplan-Meier analysis was performed on relative complete response rates after therapy and the log-rank test was used to compare them between treatment groups. $P < 0.05$ was considered statistically significant.

Results

Response

The relative complete response rate of patches treated in this study is shown in figure 1:

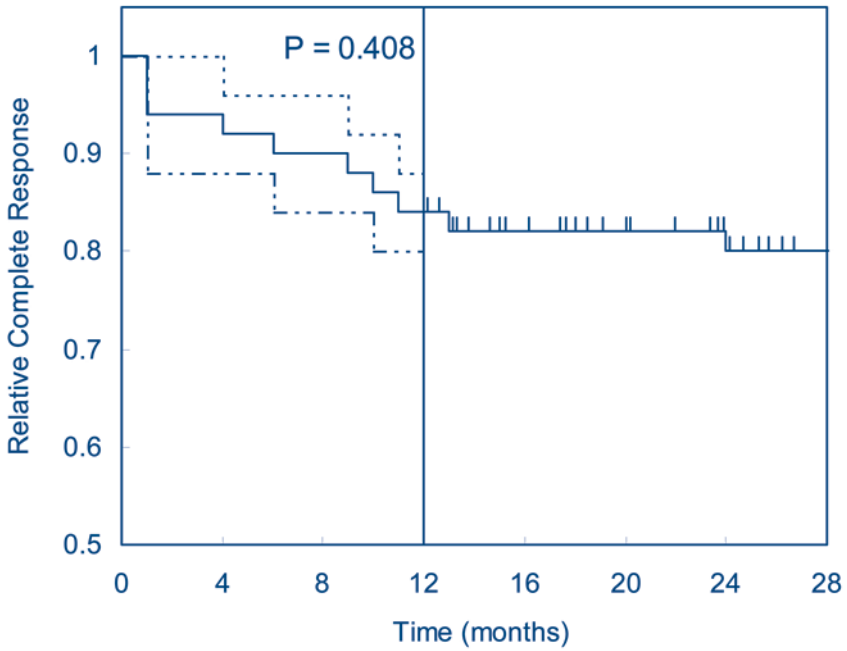

Figure 1: Kaplan-Meier curves for disease-free rates for relative complete response of Bowens disease following ALA-PDT using a single light fraction of 75 J cm^{-2}, 4 hours after the application of ALA(——-——-), a two-fold illumination scheme of 20 + 80 J cm^{-2} 4 and 6 hours after the application of ALA. (----------), (n=25 in each group). The combined disease-free rate for all Bowen's patches (————), (n=50); tick marks show maximum lesion follow up, the number of lesions remaining at the end of the period shown is 14/50. Statistical significance between illumination schemes is tested for one-year follow up (P=0.408 log rank test).

In the single illumination group a complete response was achieved in 20 patches (80%) at 12 months. Recurrent Bowen's patches were excised in two cases (after a FU of 7 and 12 months, histology showed Bowen's). Four recurrent patches were retreated with PDT and responded. In the two-fold illumination group complete response was recorded in 22 patches (88%) at 12 months. Three recurrent patches were retreated with PDT and have resulted in complete response to date. One recurrent patch on the face was excised after 24 months, histology showed Bowens disease that extended deep into the hair follicles. In each treatment group re-treated patches are not included in responders group. We found a better response of patches treated in the two-fold illumination group compared to the single illumination group, 88% vs 80%, but this increase in response did not reach statistically significance (p=0.408 log rank test). Pooling the data from each illumination group the average CR in all patches was 84% at one year follow up. The progression of clinical response of all lesions in time after 12 months is shown in figure 1 (see above). Patches with

follow up between 12 and 28 months are shown as ticks in the Kaplan–Meier curve. Overall the complete response is 80% with a mean and median follow up of 24 months. Recurrent patches were located on the leg (2), face (3), trunk (3) and arm (2). The diameter of patches that recurred varied from 5 to 23 mm, with an average diameter of 12 mm. While the numbers of recurrent patches is small, recurrence to PDT does not seem to be related to location or size.

Pain and discomfort

In the single illumination group none of the patients complained of pain during therapy. In the two fold illumination group 5 patients complained about pain in the treatment of 6 patches. Lidocaine without epinephrine was used in 4 patches. While all patients suffered some degree of discomfort all completed therapy.

Healing time

At the first follow up visit all patches were completely healed, patients reported that all patches healed completely within 3 weeks after therapy.

Cosmetic outcome

The cosmetic outcome in both groups was good, determined both by dermatologist and patient. In 2 patches in the two-fold illumination group the cosmetic outcome was scored to be fair due to persistent hyper-pigmentation at 12 months.

Comments

Response

We studied the response of Bowens disease to ALA-PDT using two different illumination schemes, with a minimum follow up of one year. PDT in a single light fraction of 75 J cm^{-2} resulted in a complete response rate of 80%. Fractionated illumination in which two light fluences (20 + 80 J cm^{-2}) are delivered separated by a two-hours dark interval resulted a higher complete response rate of 88%. This increase in response rate is not statistically significant (P=0.408). It is however of the same order of magnitude that we have previously observed in sBCC(12), where a CR of 97% at one year is significantly higher than that following PDT with a single light fraction 89% (P=0.002). The response rate of Bowens disease is lower than the

corresponding response rate for sBCC for both the single and 2-fold illumination scheme[12]. While these differences are not statistically significant (P=0.25 and P=0.09 respectively) possibly due to the relatively small number of patches in the present pilot study. It may be regarded as an illustration of the general observation that Bowens disease responses less well to all therapy modalities as the recurrence rate of the gold standard surgery is also 19%(2). Both sets of clinical response data are supported by our pre-clinical animal data in which we have shown that PDT with a two-fold illumination scheme results in significantly more severe acute response which is accompanied by deeper damage seen in histology (6-10). In Bowens disease we consider deeper damage to be an advantage partly because of the follicular involvement that is often observed. Repeated treatment is known to achieve better complete response rates in other skin conditions. Repeated treatment in Bowens disease showed higher response rates in earlier published studies (3,5,13). In our study 7 recurrent patches were retreated with PDT and responded completely in all cases. We believe optimising PDT in the first instance is superior to repeating treatment. In comparing reported response rates critical regard should be paid to both frequency of treatment and follow up time.

Use of different total fluence

It is important to recognise that the cumulative fluence in each of the illumination groups is not equal. 75 J cm^{-2} was delivered in a single light fraction compared to 100 J cm^{-2} in the two-fold illumination scheme. This is a direct consequence of our intention to deliver a large fluence in the second light fraction since we have shown that this is essential for an optimal response(10). A number of pre-clinical studies have shown that photobleaching of PpIX limits the PDT dose that can be delivered in a single light fraction at fixed fluence rate (14). The relationship between response to ALA-PDT and fluence has not been systematically investigated in the clinic. Only Oseroff has emphasised the importance of light fluence in the treatment of sBCC by topical ALA-PDT (15), and this regards the delivery of high light fluences at high fluence rate. He reported that a CR rate of 95% after 200 J cm^{-2} (at a fluence rate of 150 mW cm^{-2}) fell to 70% after 150 J cm^{-2} (16). Most other investigators have applied both lower light fluence (rate) and cumulative fluences both below 75 J cm^{-2} and above 100 J cm^{-2}, with little evidence for a correlation between fluence and response. There is some clinical evidence, albeit limited, that PpIX photobleaching limits the response to a single light fraction following ALA-PDT of AK (17).

Light sources

As described above we have previously shown that there is no significant effect of light source on the enhanced response to ALA-PDT using light fractionation with a 2 hours dark interval (12). A variety of light sources have previously been used for PDT for Bowens disease (3, 12, 18-20) and there are conflicting data on the influence of the light source spectral output and fluence rate used in ALA-PDT (21,22). While the present study is insufficiently powered to investigate the effects of light source we do not believe that the light source plays a significant role in the response of tissues to ALA-PDT using light fractionation. Our own data in sBCC (12) and recent data in ALA-PDT of AK (23) support this conclusion.

Adverse events and cosmetic outcome

In our pre-clinical animal studies investigating the two-fold illumination scheme we have found significantly deeper damage that is accompanied by an inflammatory infiltrate in the dermis (10). In some cases we have observed scarring in normal mouse skin. We were therefore careful to note the incidence of adverse events and cosmetic outcome in patches illuminated using the two-fold illumination scheme. Cosmetic outcome was good in all but 2 patches due to persistent hyperpigmentation. This result is similar to our previous clinical studies involving the two-fold illumination scheme in sBCC were we did not find a significant difference in cosmetic outcome between a single and two-fold illumination scheme (11, 12). Patients treated with a two-fold illumination scheme tend to have more discomfort. Patients were asked for adverse events and healing time and reported only discomfort like edema, erythema and minor crusting healed within three weeks. Only patients in the two-fold illumination group required local anesthesia which was sufficient to relieve the pain. Comparing these results to other treatment modalities PDT using the two-fold illumination scheme can be regarded as an attractive treatment option. In patches treated with cryotherapy adverse effects such as ulceration are seen and healing time is sometimes long (3,24). Reported response rates following curettage & cautery (25) may be high but the healing time is much longer than that associated with PDT.

Conclusion

Two-year response rates to PDT and conventional surgery are comparable and in our view PDT may offer the best treatment option for Bowens disease. Surgery may be contraindicated for general health reasons (e.g. mean age of the current study is 74). Also Bowens disease is often located on areas that are difficult to treat such as the lower legs and the face. For patches in these areas, and in particular for the larger ones, there is a significant need for an alternative to surgery. The present pilot study shows the potential of light fractionation for enhancing the response of Bowens disease following ALA-PDT by the same order of magnitude that we have shown in sBCC and illustrates the need for a larger, suitably powered, study to determine if this is a statistically significant effect.

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Chapter 4

In vivo fluorescence pharmacokinetics of PpIX fluorescence in a two-fold illumination scheme of ALA-PDT for superficial BCC's

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Abstract

Background: Light fractionation in ALA-PDT using a small first fraction and a substantial larger second fraction separated by a two hours dark interval, has been shown to enhance the response in several animal models and in superficial non melanoma skin cancer. PpIX fluorescence kinetics has been studied to date with limitations, but showed the improvement is not due to an increased amount of PpIX.

Methods: We studied 32 superficial basal cell carcinomas, 7 control lesions and 25 treated lesions. The treatment scheme we used: after topical 20% ALA, 20+80 Jcm $^{-2}$ separated by a two hours dark interval, red light. Both surface and microscopic PpIX fluorescence were studied. Biopsies were taken just before the second illumination (6 hours after ALA application, or two hour after the first illumination). Each biopsy was immunohistochemically stained for the proliferation marker Ki-67.

Results: A large variation in PpIX fluorescence was found between the control and the treated biopsies as well as within a biopsy. Despite the variation a reasonable correlation was found between the two techniques, surface and microscopic fluorescence, we used. There is no trend in depth of PpIX in the studies samples. Re-appearance of fluorescence is found just before the second illumination. The distribution of positive stained cells for Ki-67 follow the PpIX fluorescence distribution.

Conclusion: Spatial microscopic PpIX in superficial basal cell carcinoma shows a wide variation between and within biopsies. PpIX fluorescence confirmed relative resynthesis in the dark interval in a two-fold illumination scheme consistent with our earlier work.

Introduction

Light fractionation using a 2-hours dark interval significantly enhances the clinical response of superficial basal cell carcinoma (sBCC) to 5-aminolevulinic acid (ALA) based photodynamic therapy (PDT) (1, 2). The increased effectiveness of this 2-fold illumination scheme was first demonstrated in a series of pre-clinical studies and optimised in the hairless mouse model (3, 4, 5, 6). Despite these encouraging results the mechanism behind the increased effectiveness has yet to be fully elucidated. We have shown that the overall amount of PpIX re-synthesised in time after the first light fraction of the two-fold illumination does not correlate with the response to the treatment (6, 7, 8). Increasing the length of the dark interval from one to two hours leads to a significant increase in visual skin response despite the fact that the amount of PpIX utilised is not significantly different (7). We have also shown that light fractionation using methyl aminolevulinate (MAL), an ester derivative of ALA, does not increase the response of normal mouse skin above that following a single light fraction (9). This is despite the fact that the application of MAL results in identical fluorescence pharmacokinetics during the two-fold illumination. A significant disadvantage of surface fluorescence measurements is that the depth distribution of photosensitiser is not fully resolved. For thin tissues such as the normal skin of pre-clinical models, the depth distribution of PpIX is normally neglected (6, 10 - 13). For thicker lesions such as basal cell carcinoma it is likely that the depth distribution is a significant confounding factor in the determination of the pharmacokinetics of PpIX. Microscopic analysis of tumour biopsies, sectioned and imaged using fluorescence microscopy, has previously been used to determine the distribution of PpIX in BCC and in other organs (14 -18). These studies have, for the majority, investigated distribution of PpIX in nodular BCC. Only one has investigated the microscopic distribution in superficial BCC after topical ALA (19). Also the spatial distribution of PpIX re-synthesis in BCC has not been investigated. For these reasons, in the present study, we have investigated the spatial distribution of PpIX in sBCC undergoing light fractionated ALA-PDT comparing both surface and fluorescence microscopy. In addition we have investigated the presence and distribution of the proliferation marker Ki-67. Ki-67 is a protein that is detected in the nucleus in phase G1, S, G2 and M of the cell cycle. Ki-67 is considered to be vital for proliferation. The number of cells with Ki-67 is used to estimate cell proliferation. In some malignancies, for example breast cancer, Ki-67 has prognostic

value. In basal cell carcinoma an average of 20% positive cells is been reported (20 - 23). A uniform distribution of Ki-67 positive cells has been reported (24) suggesting an equal proliferation in neoplastic cells over the bulk of the tumour and in tumour islands. A more recent study by Tilly et all found proliferate activity restricted to the periphery of tumour nests in superficial and nodular basal cell carcinomas (25). In aggressive and recurring basal cell carcinomas Ki-67 expression has been shown to be higher (26). Vidal et al studied the expression of Ki-67 among others in BCC in response to topical imiquimod treatment. They found pre-treatment a high percentage positive Ki-67 cells (39%) and showed no significant modification during imiquimod treatment (27). Here we have compared the distribution of Ki-67 with the distribution of PpIX in sBCC undergoing light fractionated ALA-PDT.

Material and Methods

Patients and ALA application

Eight patients, diagnosed with multiple superficial basal cell carcinomas, were recruited to investigate the superficial and microscopic distribution of PpIX during light fractionated ALA-PDT. The design was approved by the ethical committee of the Erasmus MC, according to the Declaration of Helsinki Principles. All patients gave informed consent. Thirty two lesions were included in the present analysis. Where possible four lesions were identified in each patient. The location of lesions was dependent on the patient but sites on the back, chest, arm, face and leg were used. One lesion was randomly designated as a control lesion to which ALA was administered but was not illuminated. The remaining three lesions underwent light fractionated ALA-PDT. Topical ALA ointment was prepared by our Hospital Pharmacy using 20 % aminolevulinic acid (FLUKA Netherlands) in Instilagel ® (Medeco B.V. Oud Beijerland, The Netherlands). The ointment was freshly prepared and stored in a refrigerator to be used within 7 days. Before application of ALA, crusts and scaling were removed using a disposable curette. Control and treatment lesions were covered with a margin of 1 cm and dressed with a semi permeable dressing (Tegaderm® 3M, Netherlands) over aluminium foil to protect the lesion from light during application of ALA.

Photodynamic therapy and biopsy collection

Illuminated lesions received light fractionated ALA-PDT using a two-fold illumination scheme in which 20 + 80 J cm^{-2} was delivered separated by a 2 hours dark interval (1). A light fluence of 20 J cm^{-2} was delivered 4 hours after the administration of ALA, at a constant uniform fluence rate of 50 mW cm^{-2}, using a 630 nm diode laser (Carl Zeiss, Oberkochen, Germany). Immediately after the therapeutic illumination lesions were re-covered with a light protective dressing for a dark interval of 2 hours. Immediately before the second light fraction, 6 hours after the administration of ALA, a 5 mm punch biopsy was obtained from the centre of each lesion. Biopsy sites were stitched, swabbed and applied with light pressure until any bleeding had stopped. The remaining lesions then received a second light fraction of 80 J cm^{-2}. A margin of at least 5 mm around each lesion was included in each treatment field. Control lesions that did not receive the first light fraction were biopsied at the corresponding time point, 6 hours after the administration of ALA. After the punch biopsy was taken control lesions were stitched and swabbed. Control lesions did not undergo PDT in this treatment session but were allowed to heal and subsequently treated using light fractionated ALA-PDT.

Superficial PpIX fluorescence imaging

Superficial PpIX fluorescence images were acquired to investigate the spatial kinetics of PpIX re-synthesis. Data were acquired immediately before ALA-application, immediately before and after the first illumination, and immediately before the second illumination. Measurements were performed as we have described previously using a custom applicator attached to a fluorescence-imaging camera (1). Images acquired at different time points were registered under translation and rotation using landmarks in the skin surrounding the lesion (e. g. hair follicles). Typically a rectangular area of 4 x 6 cm is imaged that includes the whole lesion and the biopsy site. Where possible the site of the biopsy was identified in each series of 3 images (acquired before and after the first light fraction and before the second light fraction). An additional image was acquired after the end of the second light fraction, used to aid the localisation of the biopsy site in the series images. A circular region of interest approximately 0.5 cm in diameter is defined and the average pixel intensity was used as a measure of the fluorescence intensity from an area of tissue that coincided with the area from which the biopsy was taken. Autofluorescence was measured before each ALA application and was subtracted from the PpIX fluorescence intensity acquired before and after each illumination.

Fluorescence microscopy

Frozen skin samples were handled under subdued light conditions. Tissue-Tec® II embedding compound (Leica, Leiden, The Netherlands) was used to mount the skin sample on the sample holder of a cryostat (Leica, Leiden, The Netherlands). Cross sections were cut and singularly mounted on glass slides (Menzel, Braunschweig, Germany). Each tumour biopsy was sectioned at three depths collecting one 20 μm section and three 5 μm sections at each depth. Tumour sections were allowed to thaw at room temperature for 30 minutes to one hour after sectioning before imaging. Very low intensity red light using a high pass 695 filter was used to localise the section and focus its image. Fluorescence images were acquired using a slow scan CCD camera (Proscan, Lagerlechfeld, Germany) mounted on a fluorescence microscope (Leitz DM RB, Leica, Leiden, The Netherlands) equipped with an N2.1 filter block. This filter combination includes a 515-560 nm band pass excitation filter and dual dichroic and long pass detection filters (RKP580 and LP590 respectively, Leica, Leiden, The Netherlands). Light from the sample was then filtered using an additional 635±5 nm interference filter (BP635, Melles Griot, Zevenaar, The Netherlands). Background and reference (KV470 filter, Schott, Tiel, The Netherlands) images were recorded for each set of fluorescence images. The fluorescence images were corrected by subtracting the corresponding background image and dividing the resulting image with the corresponding KV470 image. All images were corrected for variations in excitation light intensity. A 120 second integration time was necessary to maximise the signal to noise ratio for the detection of PpIX. Longer integration times reduced the signal to noise due to PpIX photobleaching during image acquisition.

Immunohistochemical staining

For each biopsy an additional 5 μm tumour cross section was cut immediately adjacent to the final section of the fluorescence analysis. These sections were analysed for the presence of the nuclear antigen Ki-67 proliferation marker using a method adapted from that suggested by DAKO (Heverlee, Belgium).

Fluorescence image analysis and assay scoring

For each frozen tumour biopsy, three sets of 5 and 20 μm sections cut more than 250 μm apart were used to determine the relative intensity and spatial distribution of PpIX. Twenty micron sections were imaged using 50x magnification and 5 μm

sections were imaged using the 400x magnification. Low magnification imaging was chosen so that the full thickness of the tumour and the underlying dermis could be acquired in a single image. High magnification sections provided a more detailed image of the boarder between tumour and normal tissue. The spatial distribution of PpIX fluorescence within the section was noted at both high and low magnification. Also within each image three areas of tumour were identified and the PpIX fluorescence intensity and its standard deviation was measured in a square region of interest. The size of these regions of interest were approximately 50 x 50 and 5 x 5 μm using 50x and 400x magnification respectively. Immunohistochemical scoring was performed without prior knowledge of the distribution of PpIX fluorescence. The overall Ki-67 score was calculated as the percentage of positively stained cells to total cells.

Results

Figures 1a and 1b show the variation in the mean PpIX fluorescence intensity, measured in sections acquired from superficial BCC, six hours after the administration of ALA in control lesions (C1-7) and in lesions (1, 2 4-25) at the end of the dark interval 2 hours after the delivery of the first light fraction of 20 J cm^{-2}, 4 hours after the administration of ALA. Panels a and b show the results from fluorescence analysis and 400x (5 μm sections) and 50x (20 μm sections) magnification respectively. It was not possible to identify a control lesion in one patient. Figure 1c shows the relationship between the average PpIX fluorescence intensity using the two magnification / sectioning techniques. There is a reasonable correlation between the fluorescence intensity recorded with each technique (R^2 = 0.86). For both analyses of the distribution in PpIX fluorescence there are large variations in PpIX fluorescence intensity between individual control and illuminated lesions. The standard deviation of PpIX fluorescence calculated from weighted average of 3 regions of interest in 3 sections per biopsy are large, approximately 20 - 25% of the average absolute fluorescence intensity in an individual biopsy. The fluorescence intensity in control biopsies is greater than that in illuminated lesions 2 h after the end of the first light fraction. There is no significant difference between the standard deviations within and between regions of interest from individual biopsies for the control and illuminated lesions. Figure 1d shows the relationship between the

average PpIX fluorescence intensity measured using the surface collection technique and the microscopic analysis using the 400x magnification. We were able to localise the biopsy site in each of the three pre-biopsy surface images in 17 of the 25 lesions that were illuminated. There is a weak correlation between these two methods of determining the PpIX fluorescence intensity (R^2 = 0.54). The standard deviations for each technique are again large.

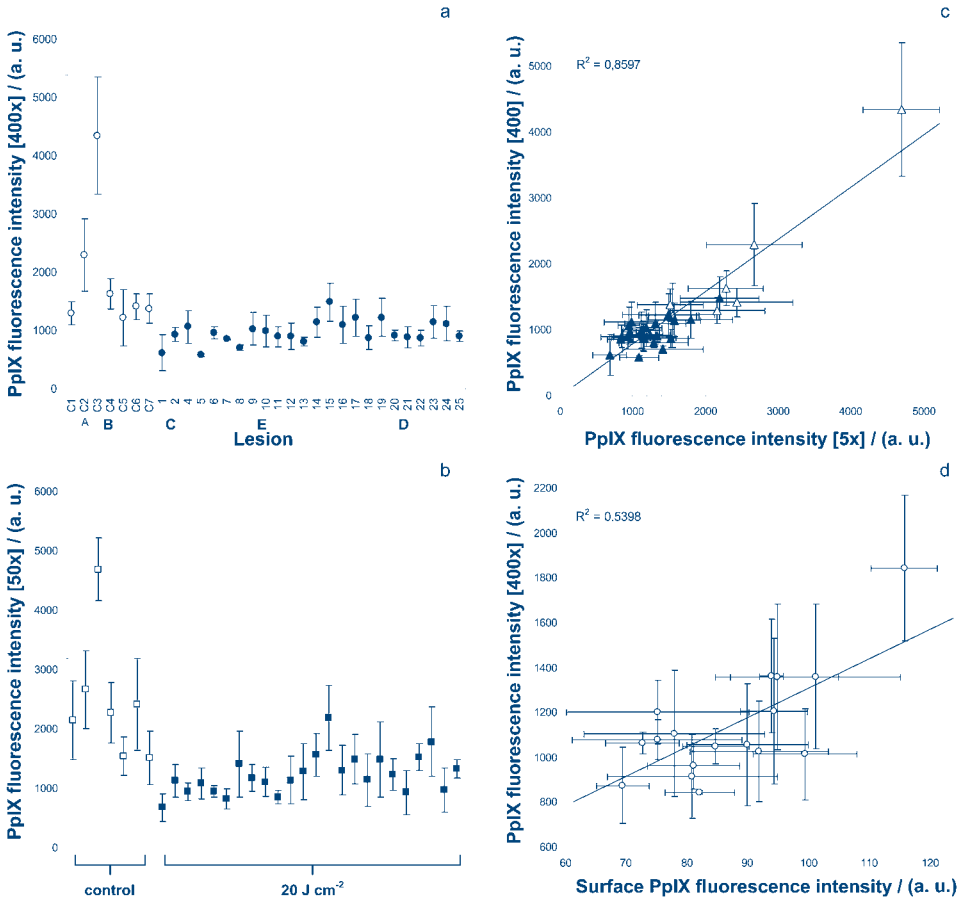


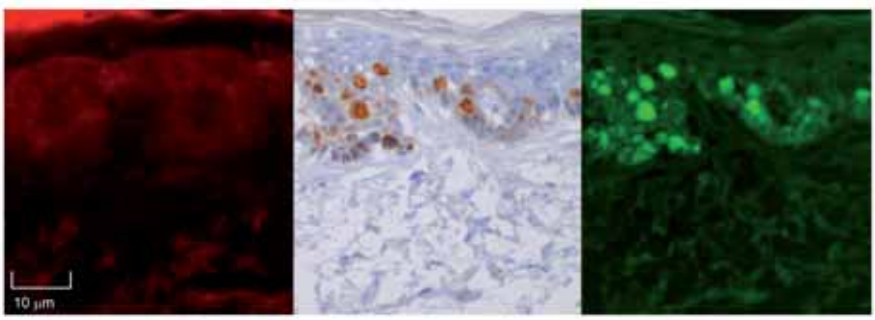
Figure 1: PpIX fluorescence image analysis during ALA-PDT of superficial basal cell carcinoma. Showing the average microscopic fluorescence intensity measured using (a) 50 and (b) 400x magnification in control (c1 – 7) un-illuminated lesions 6 hours after the application of 20% ALA and in treated lesions (1-25) 2 hours after 20 J cm^{-2}. The correlation between the microscopic fluorescence intensity measured using 50 and 400x magnification is shown in panel (c). Panel (d) shows the correlation between the surface and microscopic fluorescence intensity. In all cases error bars represent standard deviations.

Figure 2 a - e shows 5 representative tumour sections that have been analysed for PpIX fluorescence distribution, (left red image) and Ki-67 positively stained cells

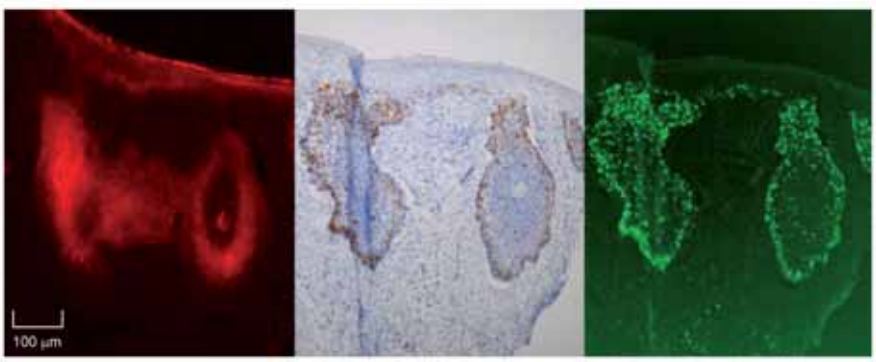
(centre and right image). The centre Ki-67 image has undergone spectral unmixing in the right hand image, to show positively stained brown cells above the blue background due to haematoxylin. Panels a and b show sections of control lesions that were biopsied 6 hours after the administration of ALA. Panels c – e show sections of lesions that were biopsied 2 hours after 20 J cm^{-2}, 6 hours after the administration of ALA. Overall, analysing all of the sections under high magnification, an average of 20% of tumour cells were positively stained for Ki-67 compared to the total number of tumour cells. There was no difference in the proliferation index between control or illuminated lesions. Panel a shows a superficial BCC that extends into the upper dermis. Tumour cells in the region show significant fluorescence that extends into the upper dermis . There is a relatively uniform distribution of PpIX fluorescence in the tumour cells that includes cells that are stained Ki-67 positive and cells that are not. Panel a also shows significant fluorescence deeper which seems to be associated with collagen fibres. Panel b shows a 50x magnification 20 μm section of a control biopsy that shows the deeper extension of BCC into the dermis. The section illustrates peripheral palissading and retraction artefact at the boundary between tumour cells and the surrounding dermis, that are characteristic of BCC. In this section there are significant differences in the distribution of PpIX fluorescence. In the island of BCC on the left of the section there is a rather uniform distribution of fluorescence. The island of BCC on the right of the section shows a peripheral distribution of PpIX in which the centre of the island shows very little PpIX accumulation. There is no obvious general trend for a depth distribution of PpIX fluorescence in BCC. Regions of high PpIX fluorescence intensity are clearly seen in islands relatively deep in the dermis. Note that the very centre of this island shows a fluorescence artefact that is probably due to fluorescence from the glass beneath the section that is exposed due to the hole in the section clearly seen in the central Ki-67 image. In this section the distribution of Ki-67 positive cells closely follows the distribution of PpIX. The interior of the island on the right shows very little or no Ki-67 positive cells while the island on the left of the sections shows a more uniform distribution of Ki-67 positive cells. Panel c shows a section of an illuminated lesion 2 hours after PDT showing the basal layer of the epidermis an island of BCC between the epidermis and a deeper lying region of tumour cells. In the superficial layers of the section there is a relatively uniform distribution of PpIX fluorescence that includes cells that are stained Ki-67 positive and cells that are not. In the tumour island and in the lower region of the image PpIX fluorescence closely corresponds

to Ki-67 positive cells. Panels d and e show in more detail the border between tumour cells and cells of the dermis. Again there is a moderate correlation between PpIX fluorescence intensity and Ki-67 positive cells in regions of tumour cells outside the epidermis where positive and negative Ki-67 cells accumulate PpIX. Again we did not observe a systematic trend for differences in the overall distribution of PpIX between control and illuminated lesions. However PpIX fluorescence in biopsies that were illuminated and imaged at 400x magnification were somewhat more diffuse than control images. This suggests that there may have been a slightly different sub-cellular distribution of PpIX 2 hours after PDT compared to the distribution of PpIX 6 hours after the administration of ALA. It is important to note that an intense fluorescence signal is observed in all sections that include the most superficial layers of the skin that include either intact stratum corneum or tumour crusts.

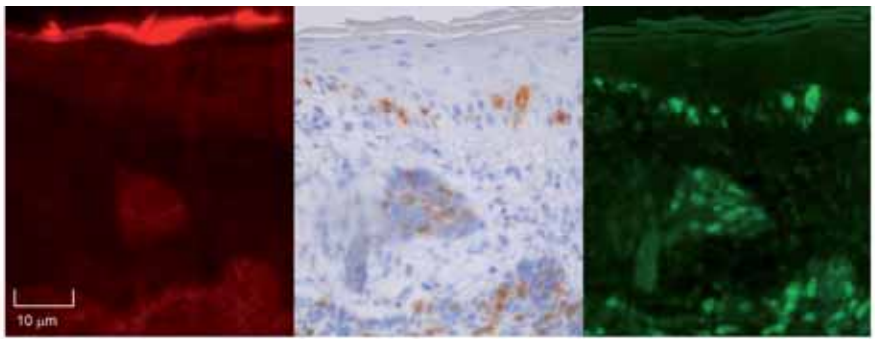
a



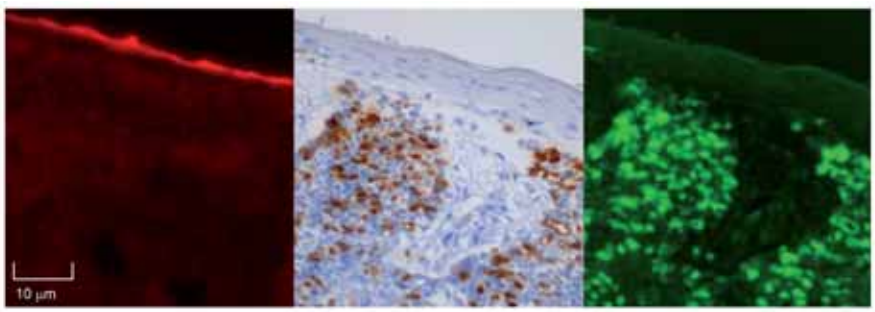
b



c



d



e

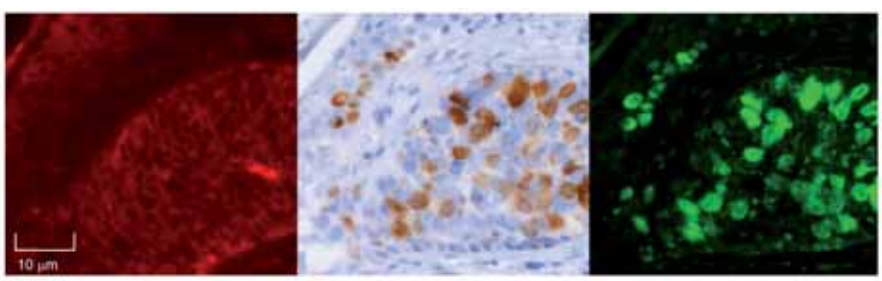


Figure 2: Representative microscopic images of sections of sBCC. Left image: PpIX fluorescence distribution, middle and right image Ki-67 staining. The middle image has been spectrally unmixed to show the distribution of Ki-67 positive cells. Panels a & b are control lesions, 6 hours after 20% ALA-application, Panels c-e are sections 2 hours after 20 Jcm^{-2}, also 6 hours after 20% ALA application. See detailed description of figure in text.

Figure 3 shows the variation in mean surface PpIX fluorescence during before and after the first light fraction and at the end of the dark interval immediately before the second light fraction. Again it was only possible to recover data for the whole sequence of images in 17 of the 25 lesions that received PDT. The PpIX fluorescence before the first light fraction varied considerably. The mean normalised fluorescence after the illumination is 48% of that before the first light fraction and rises to 63% during the 2 hours dark interval. This compares with a mean surface fluorescence intensity in control, non-illuminated lesions, that is 119% of the average immediately prior to the first light fraction, 4 hours after the administration of ALA. During PpIX fluorescence photobleaching varied widely, despite fixed illumination parameters. The average extent of photobleaching of 47% during the first light fraction is similar to that we have found in our pre-clinical studies. Also shown is the average fluorescence intensity measured using from the corresponding biopsy sections as a relative comparison.

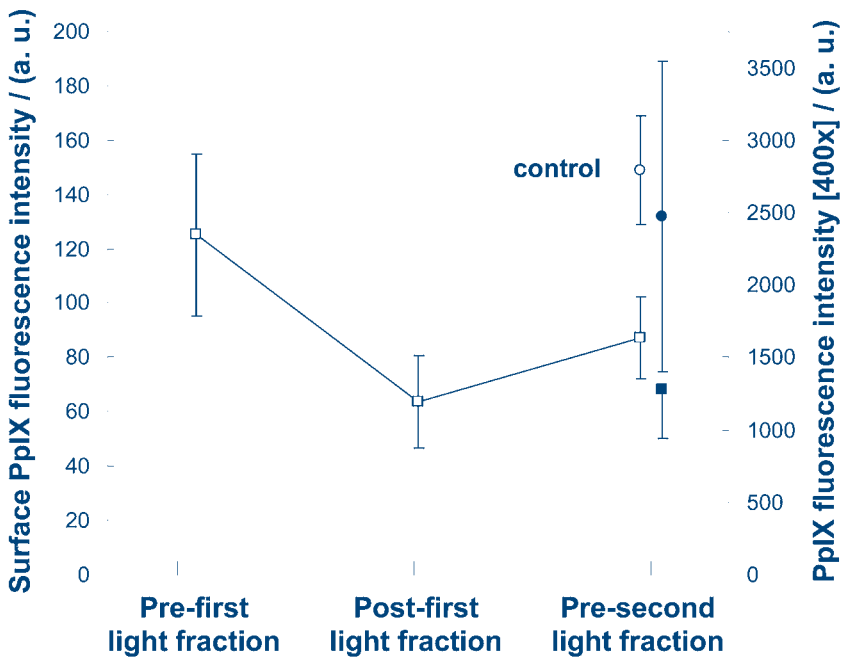


Figure 3: The kinetics of superficial PpIX fluorescence intensity in sBCC during ALA-PDT with a two-fold illumination scheme (open squares) delivering 20 J cm^{-2} and the re-synthesis during the 2 hours dark interval between light fractions and the PpIX fluorescence in un-illuminated control lesions (open circles). The corresponding average microscopic PpIX fluorescence intensity (closed symbols) is shown for comparison.

Discussion

The purpose of this study was to investigate the distribution of PpIX in superficial BCC, during light fractionated ALA-PDT, using fluorescence microscopy. We found wide variations in the average intensity of PpIX fluorescence in biopsies of control lesions and in biopsies of lesions 2 hours after illumination with 20 J cm^{-2}. We also found wide variations in fluorescence intensity within individual sections of biopsies. The large variability in fluorescence intensity measured in adjacent tumour sections is likely to be a consequence of the difficulty of making sections of reproducible thickness. Also since tissue sectioning is known to disrupt cell and organelle membranes it is possible that PpIX bound at lipophilic sites within the tissue localises in different environment(s) that induce unpredictable variations in fluorescence quantum yield of PpIX. This is a particular problem for thin (5 μm) sections. Given these large standard deviations on average fluorescence intensities we found a reasonable correlation between the two different methods of performing fluorescence microscopy, as shown in figure 1c. The biopsy average PpIX fluorescence intensity determined using 20 μm sections and 50x magnification was similar to that determined using 5 μm sections and 400x magnification. This is an encouraging result that adds some weight to the use of semi-quantitative fluorescence microscopy as a method of determining PpIX intensity. Large variations in microscopic PpIX content after ALA administration have been observed previously in sBCC (19). Martin et al attributed these variations to differences in ALA penetration through intact stratum corneum and ALA transport within individual tumour. The transport of ALA in large nodular tumours is clearly an important parameter that influences the synthesis of PpIX at depth. However in the present study we have shown for superficial BCC, where ALA penetration and transport are unlikely to be limiting factors, there remain regions of tumour that accumulate and re-synthesise relatively small amounts of PpIX. It is also clear that regions of low PpIX fluorescence accumulation are not located at the base of superficial tumours and high PpIX fluorescence is often observed at the periphery of tumour islands at the base of the tumour, as seen in figure 2b. Clearly there are other more important mechanism(s) that influence the synthesis of PpIX in sBCC when high concentrations of ALA are present. As long ago as 1997, Peng et al were able to review the literature and conclude that PpIX is preferentially synthesised in tumours and in rapidly dividing cells (28). Our present data support these general conclusions. More rapidly dividing tumour cells at the base of s-BCC,

that are Ki-67 positive synthesise relativity more PpIX. The underlying mechanism behind the differential synthesis is complex but though to be a consequence of the relative activity of enzymes within the biosynthetic pathway of heme, in particular porphobilinogendeaminase and ferrochelatase (29). In the present study we have shown that even in s-BCC, where the local availability of ALA is not a limiting factor, significant numbers of tumour cells synthesise relatively low amounts of PpIX. Reduced PpIX synthesis, at depth, where the concentration of ALA is limited has been suggested as a important factor in the reduced response of nodular BCC following ALA-PDT. Our results suggest that the ability of cells to synthese PpIX is also an important consideration. In this case modification of heme biosynthesis, particularly in cells that do not synthese sufficient PpIX, seems an attractive option (30).

The large spatial variations in distribution of PpIX fluorescence, in particular the variations associated with depth, illustrate the challenge for fluorescence detection of s-BCC, that is frequently advocated a method of optical diagnosis (31-34). Recently Stenquist (35) published data describing the use of fluorescence imaging to demarcate the boundaries of basal cell carcinoma during Mohs' micrographic surgery. A mapping precision of +/-3mm was estimated The authors uncorrected in-vivo fluorescence imagining.

As noted above we observe a fluorescent layer in the most superficial layers of the stratum corneum, the origin of this fluorescence is unknown and may be an artefact of the sectioning process.

The fact that we have found similar spatial distributions of PpIX, 2 hours after the first light fraction of 20 J cm^{-2}, compared to un-illuminated control biopsies confirms our recent pre-clinical findings that there is no significant influence of PDT on the ability of cells or tissue to re-synthesis of PpIX and that there is little evidence for a systemic component of PpIX re-synthesis (36). The average amount of PpIX that is re-synthesised during the two hours dark interval after the first light fraction measured using fluorescence microscopy and superficial imaging are consistent with those that we have observed previously (1, 2). The present study does however illustrate the inherent weakness of spatial averaging of tissues in which there is large variation in the spatial (re-) synthesis of PpIX.

We are continuing to investigate the mechanism behind the response of tissues to light fractionated ALA-PDT. With regard to the underlying mechanism it is important to note that all of the illuminated lesions investigated in the present study all showed

complete response to light fractionated ALA-PDT 24 months after therapy. This is in spite of the significant variations in microscopic PpIX fluorescence intensity measured immediately before the second light fraction. While the mechanism behind this increased response is not yet completely clear we have recently shown that the average kinetics of superficial PpIX fluorescence may be misleading (37). Our data in normal mouse skin indicate that the microscopic distribution of PpIX re-synthesis after the application of ALA is an important aspect in the response of tissues to light fractionated ALA-PDT.

In conclusion we have measured the spatial microscopic distribution in PpIX in s-BCC and we have shown the wide variations between tumour cells within and between tumour biopsies. We have confirmed that the relative re-synthesis of PpIX after PDT is consistent with that we have recently found in pre-clinical models. We have shown that highly proliferating normal and tumour cells synthesise relatively more PpIX and that this corresponds with ki-67 staining. We have shown that even in s-BCC are significant regions of tumour cells that do not synthesize PpIX even when there is sufficient ALA availability.

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Chapter 5

Apoptosis markers in ALA- PDT treated superficial basal cell carcinomas using a single and a two-fold illumination scheme

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Manuscript in preparation

Abstract

Background: ALA-PDT is an established therapy modality for sBCC. The mechanism of cell death in PDT is partly due to apoptotic processes. We have shown that fractionated ALA-PDT leads to higher complete response rates in superficial BCC's , AK and Bowens disease. In search to enlighten this increased response as well as to elucidate the PDT mechanism we investigated some apoptotic markers.

Methods: 43 histological proven sBCC's were included: 7 control lesions, 10 lesions treated with a single dose of 75 Jcm^{-2} and 25 lesions treated with a fractionated regime (20 + 80 Jcm^{-2} separated by a two hours dark interval). One lesion in one patient was treated with the fractionated regime and serial biopsied, 6, 12 and 24 hours after the completed treatment. In the control lesions a biopsy was taken after 6 hours of ALA application. In the single illuminated lesions a biopsy was taken after 2 hours after 75 Jcm^{-2} .In the fractionated treated lesions a biopsy was taken after 2 dark hours after an illumination of 20 Jcm^{-2}. In all treated lesions ALA was applied for 4 hours, a fluence rate of 50$mWcm^{-2}$ and a diode laser system as light source was used. Immunohistochemical staining for Bcl-2, Bax, caspase 3 were done in frozen sections.

Results: In almost all biopsies positive stained tumour cells for Bcl-2 were found. In the staining for Bax in control lesions some positive tumour cells were found. In the majority of biopsies after 20 J cm^{-2} positive cells for Bax were shown. After 75 J cm^{-2} also positive cells for Bax staining were found. In all illuminated lesions an upregulated expression of caspase 3 was shown.

Conclusion: Early, after two hours, after a 75 J cm^{-2} and after a small dose of 20 J cm^{-2} apoptotic markers show altered expression indicating apoptosis is occurring. Apoptosis induced by the mitochondrial pathway plays a role in cell death in ALA-PDT in sBCC.

Introduction

Photodynamic therapy (PDT) is gaining acceptance in the treatment of superficial basal cell carcinoma (sBCC). PDT relies on the use of an appropriate photosensitiser which accumulates selectively in tumour cells and illumination with an appropriate wavelength of light. The generation of relative oxygen species results in tumour destruction. In dermatology, porphyrin pre-cursors, aminolevulinic acid (ALA) or methyl-aminolevulinate (MAL) are used as photosensitiser-precursors and are combined with illumination using red light . The production of reactive oxygen leads to a complex interplay between direct cytotoxicity to tumour cells and secondary damage to the tumour and adjacent tissue. Cell death can be due to necrosis and / or apoptosis. Necrosis can be considered as a passive process associated with cellular injury inciting an inflammatory response. Apoptosis is an active process which involves a cascade of cellular changes leading to clear tumour cells. Apoptosis is generally accepted to play an important role in the response to PDT (1) and has been shown to occur in many cancer cells in vitro and in tumours in vivo. The dose delivered during PDT is known to be an important factor in determining the balance between apoptotic and necrotic cell death. Treatment schemes that utilise high doses of drug and light (i.e. high fluence or low fluence rate) are known to induce necrosis. Low doses of PDT, soon after the administration of photosensitiser and / or low light fluence and/or high fluence rate have been shown to preferentially induce apoptosis (2). In normal skin members of the Bcl-2 family are expressed in different layers and in different amounts. The family is considered to exist of pro and anti apoptotic members. For example Bcl-2 inhibits apoptosis while Bax promotes apoptosis. Caspase 3 is known as the executor caspase in the apoptotic process.Our optimised light fractionated illumination scheme for ALA-PDT involves the delivery of relatively low PDT dose (20 J cm^{-2} at 50 $mWcm^{-2}$) 4 hours after the administration of ALA. (3,4,5). Delivering higher doses in this first light fraction ≥ 50 J cm^{-2} removes the therapeutic effect of light fractionation (4,6). With PDT dose dependent effect in mind and the aim to investigate the mechanism of cell death at early time points after ALA-PDT we investigated the induction of apoptotic markers in superficial basal cell carcinoma. We report on the presence of apoptotic markers Bcl-2, Bax and caspase 3 in human sBCC biopsies acquired 2 hours after high dose (75 J cm^{-2}) and low dose (20 J cm^{-2}). 6 hours after the ALA-application undergoing light fractionated PDT. We have also investigated the induction of apoptosis over longer time scales in sBCC by performing serial biopsies in a single lesion.

Patients, Materials and Methods

Lesions

Forty three histological proven sBCC's were included in this study. All patients gave informed consent. The study design was approved by the ethical committee of Erasmus MC according to the declaration of Helsinki principles.

ALA application

After lesion preparation using a disposable curette to remove gently crusts and scalling 20 % ALA was applied with a margin of 1 cm on all lesions. The ALA ointment (20% ALA (FLUKA, Zwijndrecht, The Netherlands in Instilagel®, Medeco B.V. Oud Beijerland, The Netherlands) is freshly prepared by our University Hospital Pharmacy to be used within 7 days. The application time in the control lesions was 6 hours, in the treated lesions 4 hours.

Treatment schemes

A 630 nm diode laser (Carl Zeiss, Oberkochen, Germany) was used as light source. Light was coupled into a 600 μm optical fibre and projected onto the lesion using a combination of lenses to assure a uniform fluence rate across the beam diameter. Lesions illuminated using a single light fraction received 75 J cm^{-2} In the light fractionated scheme 20 + 80 J cm^{-2} were delivered separated by a two hours dark interval. All lesions were illuminated with a constant measured fluence rate of 50 $mWcm^{-2}$.

Biopsies

In the control lesions (n=7) a biopsy was take 6 hours after ALA-application. Control lesions did not undergo PDT in this treatment session but were allowed to heal and subsequently treated using light fractionated ALA-PDT. In the single illuminated lesions (n=10) biopsy was taken two hours after the treatment. In the fractionated regime biopsy (n=25) was taken just before the second illumination, 6 hours after ALA-application, two hours after the first illumination of 20 J cm^{-2} . In one patient serial biopsies were taken 6, 12 and 24 hours, after a fractionated illumination.

Immunohistochemical staining

For each biopsy three 5 micron tumour cross sections were cut. These sections were

analysed for the presence of the oncoprotein Bcl-2, and Bax using a method adapted from that suggested by DAKO (Heverlee, Belgium). All sections were microscopically scored for the presence of positive stained cells to Bcl-2, Bax and caspase 3 using 40 times magnification by three of the authors (EdH, RdB DR). Results are noted : absent (-), present (+) or abundant (++).

Results

A descriptive analysis is presented, illustrated with representative images.

Bcl-2

In all control biopsies a positive staining present and abundant present, was seen for Bcl-2 in the tumour. In the fractionated treated lesions (T1-25) in 10/25 Bcl-2 staining was abundantly positive, in 12/25 only a relatively small number of positive tumour cells (scored present) were seen in a pattern like a normal epidermis. Due to artefact in sectioning 3 biopsies were not suitable for analysis. In the single illuminated lesions at 2h (S1-10) 2/10 showed abundant Bcl-2 positive staining in tumour cells , 8/10 were scored present. The serial biopsies showed significant amounts of edema and a small number of Bcl-2 positive tumour cells in all three specimens 6, 12 and 24 hours after illumination.

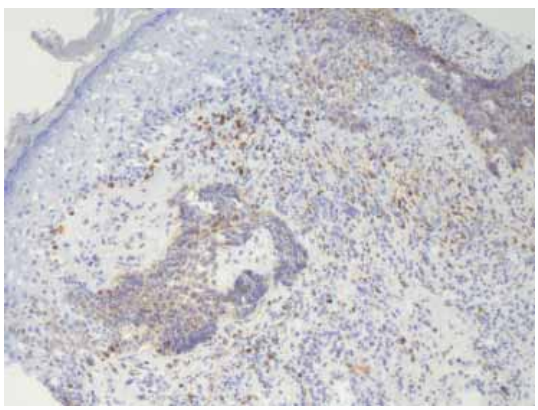
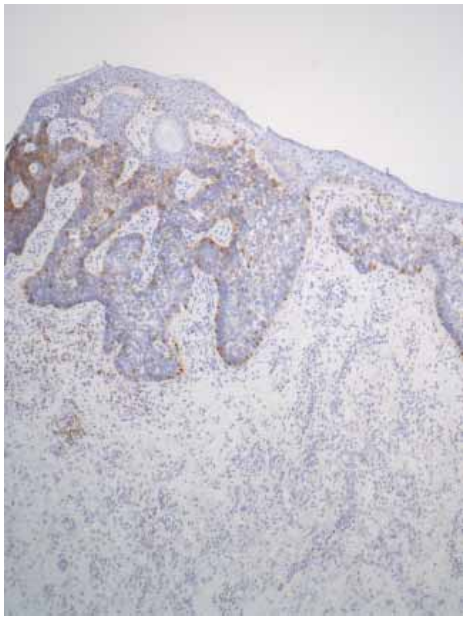


Figure 1a shows a lesion 2 hours after illumination with 75 J cm^{-2} stained for Bcl-2. Positive Bcl-2 staining is clearly seen in tumour cells and in the basal layer of the epidermis.



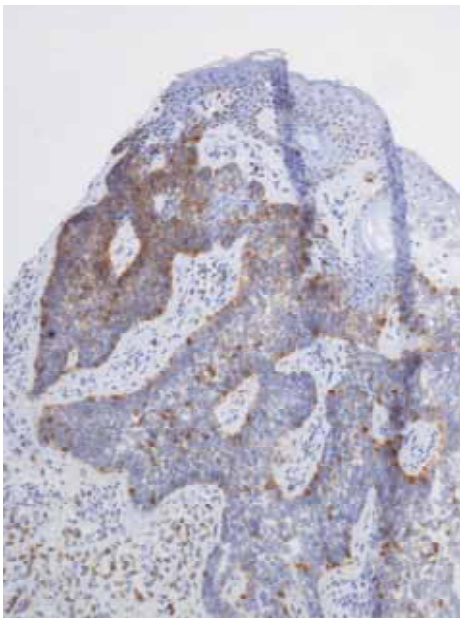


Figure 1b1-2 shows a lesion 2 hours after 20 Jcm^{-2},just before the second illumination in a fractionated illumination scheme stained for Bcl-2. Positive staining for Bcl-2 is clearly shown in the tumour cells and in the epidermis.

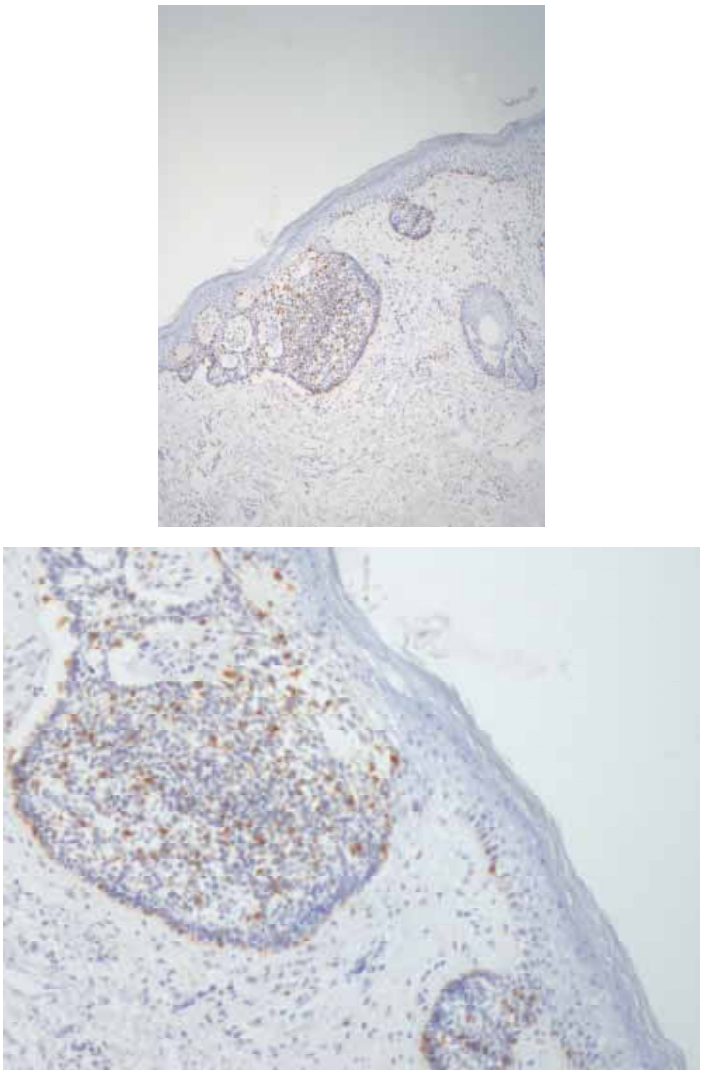


Figure 1c1-2 shows a lesion after 20 Jcm^{-2} ,just before the second illumination in a fractionated illumination scheme stained for Bcl-2. Positively staining for Bcl-2 in tumour cells are shown. The epidermis also shows positively stained cells

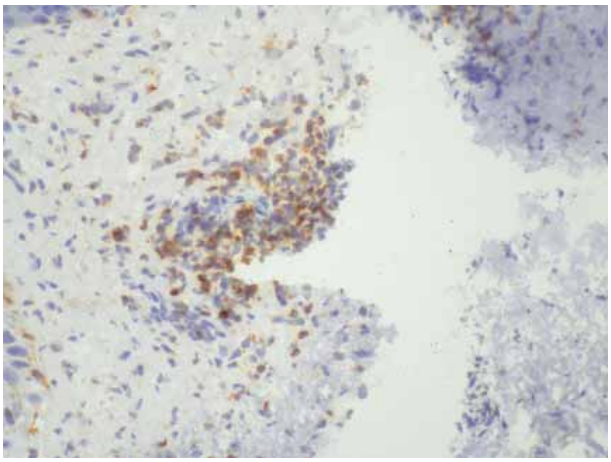
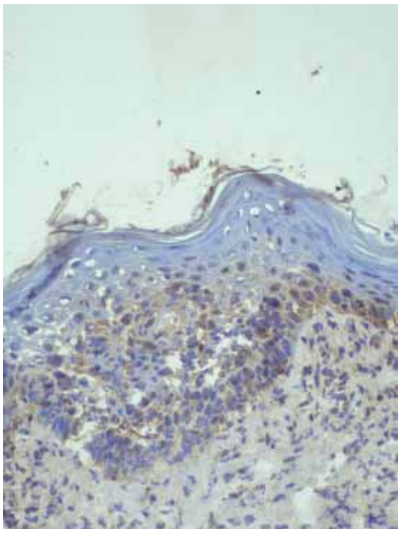


Figure 1d Shows a control lesion after 6 hours of ALA-application stained for Bcl-2. Positive stained cells for Bcl-2 in the tumour are shown.

Bax

In the control lesions 30% showed scattered positive staining. In 80% of the fractionated treated lesions (T1-25) positively stained cells were found in the sections, mostly in small groups scattered (60%) but also in the basal cell carcinoma cells (40%) and mostly in the epidermis (12%). Sometimes in all layers of the skin as well as in tumour cells positive staining was seen. In the single illuminated (S1-10) biopsies in 4/10 positive staining was found in the tumour in 4 other biopsies some positive staining cells for Bax were found scattered in the biopsy .In the serial biopsies positive tumour cells were found at 6 and 12 hours after fractionated ALA-PDT.



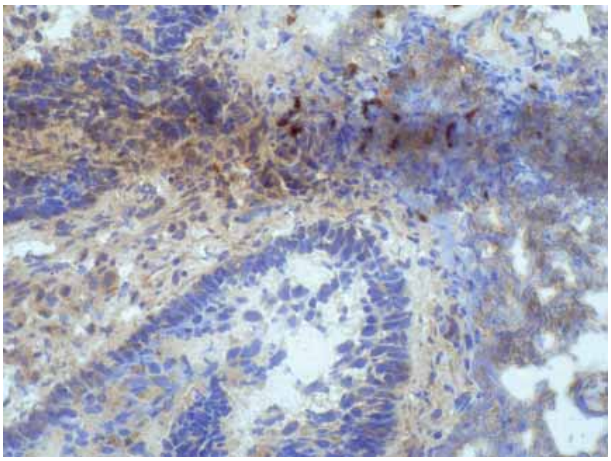


Figure 2a1-2 show a control lesion , 6 hours after ALA application stained for Bax. Some positive stained cells for Bax are shown, tumour cells are mostly not positively stained.

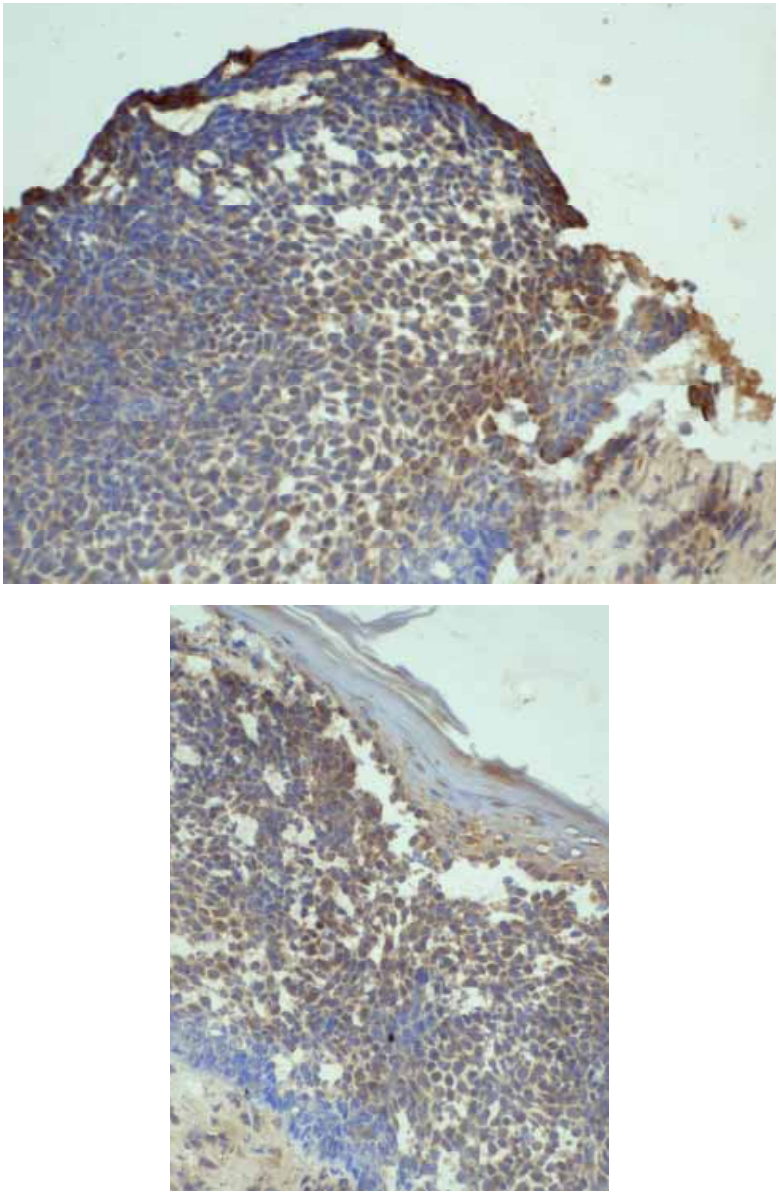


Figure 2 b 1-2 shows a lesion after 20 J cm^{-2} , just before the second illumination in a fractionated illumination scheme stained for Bax. Positive tumour cells for Bax are shown. Some positive cells for Bax are clustered in the periphery of the tumour.

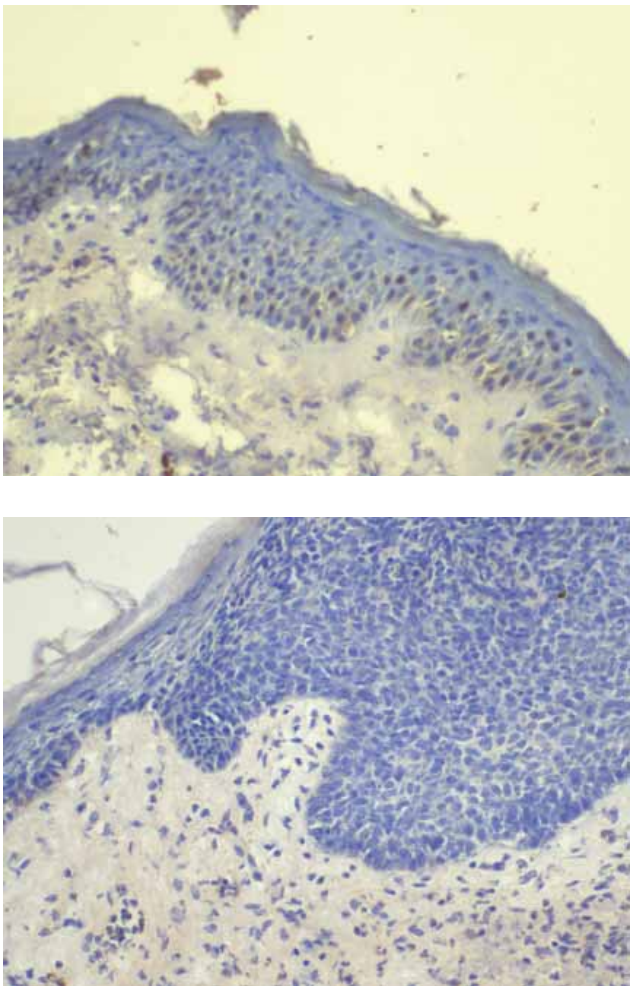


Figure 2 c1 shows a lesion 2 hours after illumination with 75 Jcm^{-2}stained for Bax. Some positive cells for Bax are shown, but clearly not the majority of cells and not in all biopsies. Figure 2c2 shows an example of a biopsy not staining for Bax.

Caspase 3

Control lesions show in 30% positive cells that are scattered throughout the tumour. In the fractionated treated lesions (T1-25) 96% of the biopsies showed positively stained cells 2 hours after 20 J cm^{-2} illumjnation, scattered, but in one third of the biopsies also clearly in the tumour. In the single illuminated lesions (S1-10) in 6/10 positive staining was shown in the tumour. In the serial biopsies at 6 and 12 hours after a fractionated treatment positive stained tumour cells for caspase 3 were found, At 12 and 24 hours some positive cells were present around the tumour.

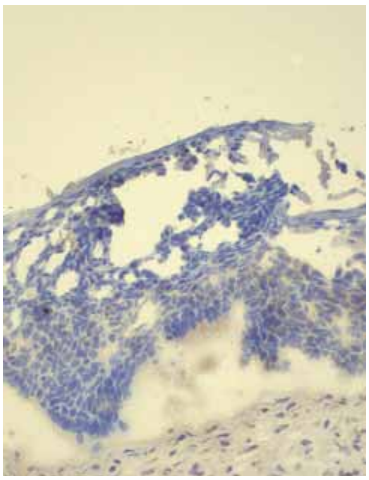


Figure 3 a shows a control lesion 6 hours after ALA application stained for caspase 3.Just some scattered lonely positive tumour cells are shown.

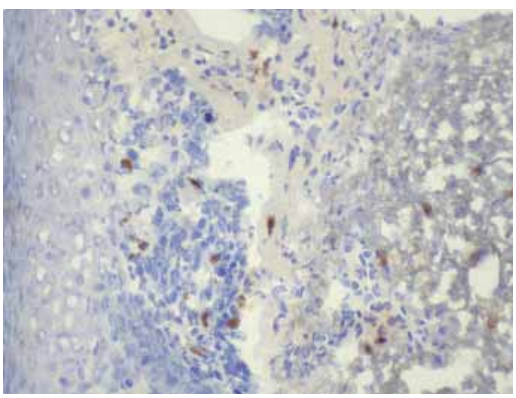


Figure 3 b shows a lesion after illumination with 75 Jcm^{-2} stained for caspase 3. Positive tumour cells for caspase 3 as well as positive cells surrounding the tumour are shown.

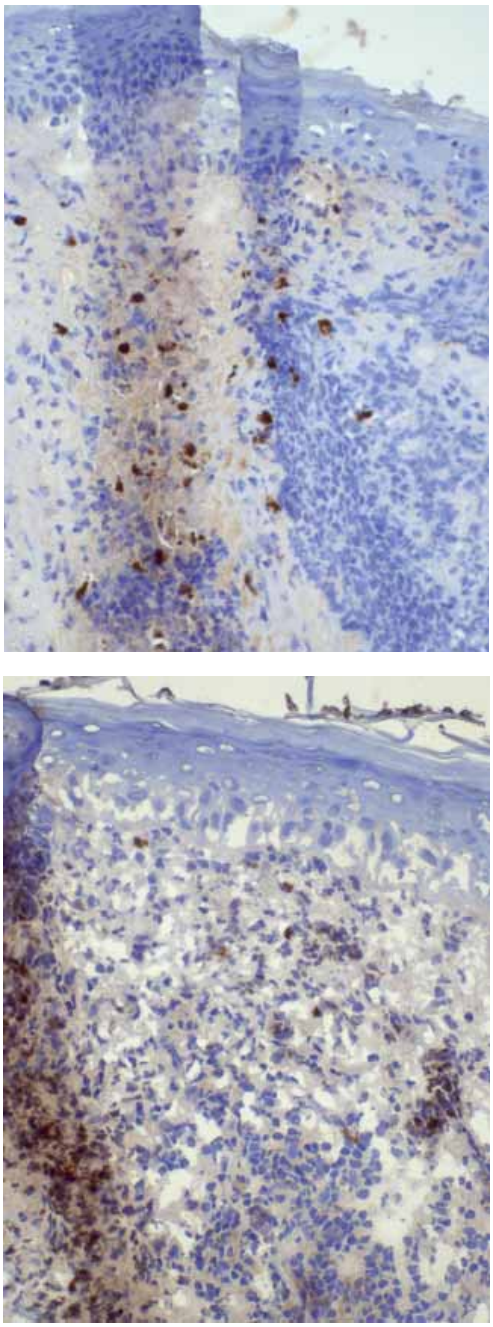


Figure 3c1-2 shows a lesion after 20 Jcm^{-2} ,just before the second illumination in a fractionated illumination scheme stained for caspase 3. Positive tumour cells for caspase 3 are shown as well as positive cells surrounding the tumour.

Table 1: immunohistochemical results in sBCC-biopsy taken at 6 hours ALA application (controls C1-7) and at 6 hours after a 20Jcm^{-2} illumination, 4 hours application time plus 2 hours dark interval (T1-25) and 2 hours after a single illumination 75Jcm^{-2} after 4 hours ALA-application (S1-10). One serial biopted sBCC at 6, 12 and 24 hours after a fractionated ALA-PDT treatment. (F1,2,3). 0 not analyzed due to artefact, - absent, +present, ++ abundantly present.

	Bcl-2 tumour	Bax	Bax tumour	Casp-3	Casp-3 tumour
C1	+	+	-	+	-
C2	++	-	-	-	-
C3	++	-	-	-	-
C4	++	-	-	+	-
C5	+	+	-	-	-
C6	+	-	-	-	-
C7	++	-	-	-	-
T1	+	+	+	+	-
T2	+	+	+	+	-
T3	+	+	-	+	-
T4	+	+	+	+	-
T5	+	+	+	+	+
T6	++	+	-	+	-
T7	0	-	-	+	-
T8	++	-	-	+	-
T9	++	-	-	+	-
T10	++	+	+	+	-
T11	++	++	+	+	+
T12	+	+	-	+	-
T13	++	+	-	+	-
T14	+	+	+	+	+
T15	0	+	-	+	-
T16	++	-	-	+	-
T17	0	+	-	+	-
T18	+	+	-	+	+
T19	++	+	+	-	-
T20	+	+	-	+	+
T21	+	+	-	+	-
T22	+	+	+	+	+
T23	+	+	-	+	-
T24	+	+	+	+	+
T25	++	+	-	+	-
S1	+	+	-	-	+
S2	+	-	+	-	-
S3	0	-	+	-	+
S4	++	-	+	-	+
S5	+	+	-	-	-
S6	+	+	-	-	+
S7	+	-	-	-	-
S8	++	-	+	-	+
S9	+	-	-	-	-
S10	+	+	-	-	+
F1	+	-	+	-	+
F2	+	-	+	+	+
F3	+	+	-	+	-

A summary of the results per biopsy per staining is shown in table one as well as a differentiation in positive staining epidermal/dermal cells to tumour cells for Bax and caspase 3.

Discussion

In sBCC we found positive staining for Bcl-2 in the tumour and in the basal layer of the epidermis. These positive patterns were not different after ALA-PDT, after either a single illumination of 75 J cm $^{-2}$ or after a light fractionated treatment scheme using 20+ 80 J cm $^{-2}$. In normal skin Bcl-2 is expressed in the basal layer. Most BCC's show positive stained cells for Bcl-2. The first reports on PDT mediated apoptosis suggested that mitochondrial damage could result in degradation or down regulation of Bcl-2 and related proteins that normally function as apoptotic suppressors (7,8).Contradictory are Bcl-2 transfected Chinese hamster ovarian cells partial resistant to PDT induced apoptosis (9). It has been reported also that over-expression of Bcl-2 can block the activation of caspases and downstream events instigated by PDT (10). Kim at al, however showed that over-expression of Bcl-2 in a human breast cell line resulted in enhanced apoptosis by PDT (11).Our findings suggest ALA-PDT does not influence the staining pattern for Bcl-2.

The pro-apoptotic member of the Bcl-2 family, Bax was also investigated in our biopsies. A scattered pattern of Bax –positive cells was found in two of our control lesions. Normally Bax is under- expressed in the basal layer of the epidermis, but up-regulated in upper layers (12). Tilli confirmed this in normal skin but she described an increased expression in BCC (13). Rosen reported contrary findings, he found a decreased expression of Bax in BCC (14).The number of our control lesion is too small to make definite conclusions on Bax in BCC. In biopsies after 75 J cm $^{-2}$ as well as in biopsies after 20 J cm $^{-2}$ Bax expression is increased. In both regimes, after 2 hours, positive tumour cells and positive cells surrounding the tumour are shown in the staining for Bax. These findings support the idea apoptosis is induced early after ALA-PDT. A relatively large cell population Bax-positive cells in BCC seems to be prone to apoptosis. The fact that in the fractionated regime, just before the second illumination the vast majority of sBCC's do show Bax-positive cells in and around the tumour suggests a small dose of ALA-PDT , 20 Jcm^{-2} at a fluence rate of 50 mWcm^{-2} , can induce a changed pattern of the pro-apoptotic protein Bax within

2 hours. In general Bax can be seen as an actor in tumour suppression by enabling cell loss through apoptosis (15).Over-expression of Bax in mammalian cells results in induction of apoptosis according to Chiu et al (16) mainly through the release of cytochrom c and activation of caspases including caspase 3. The consequences of our findings in relation to the increased response in fractionated ALA-PDT compared to a single illumination scheme clearly need further investigations. It would be interesting to observe expression of Bax in time in the same group after PDT. It can be regarded as a shortcoming of our study we only took one specimen of most lesions. Only in one patient we took serial biopsies. In these biopsies a decreasing Bax expression was seen in time. This may be due to an ongoing apoptotic response to PDT in time. But these data are not sufficient to make definitive conclusions. In a human leukaemia cell line (HL60) ALA-PDT has been shown to deregulate the expression of Bcl-2 and Bax within 2 hours after PDT (17). We did not observe a decrease in expression of Bcl-2. And contrary to Grebenova we observed increased expression of Bax as described above. The fact we can not confirm the results of Grebenova may be due to a leukaemia-cell line used compared to in vivo obtained histology from sBCC in humans.

In our control biopsies caspase 3 positive cells are not observed. Two hours after an illumination with 75 Jcm^{-2} as well as after a small dose of 20 Jcm^{-2} tumour cells positive for caspase 3 are found. In the biopsies two hours after an illumination with 20 Jcm^{-2} many positive cells for caspase 3 are shown surrounding the tumour within 2 hours, this pattern is not seen in the biopsies two hours after 75 J cm^{-2}. Caspase 3 expression is apparently up-regulated at this early time point. This finding is in agreement with the literature ; the group of Peng observed within 15 minutes release of cytochrom c and within 30 minutes caspase 9 and 3. Four hours after PDT using hexyl-ALA in a human T-lymphoma cell line 80% of cells show apoptotic features in short time. In this study both caspase dependent as caspase independent apoptotic pathways seem to be activated (18). The obtained results in caspase 3, the executor caspase, are concordant in our data to the data published by Grebenova and others (16,18-21). A rapidly increasing expression of caspase 3 is seen in almost all biopsies in our data 2 hours after a single small dose of ALA-PDT suggesting early apoptosis induced by ALA-PDT. In cell line an increase in caspase 3 expression was observed within 1 hour which declined in time (17). In the above mentioned serial biopsies of one patient we found a decline in caspase 3 after 12 and after 24 hours. But again this in only one patient. The just

mentioned results in literature are all obtained in different material to sBCC's and may be not applicable to sBCC after ALA-PDT but support strongly mitochondrial apoptosis is involved in PDT mediated cell death. Although we only investigated and described staining of a limited number of biopsies a rapid and clear apoptotic process is revealed. For understanding the mechanism in PDT a tissue response can not be extrapolated from cellular obtained results due to the underlying cooperation between tumour, sensitiser, oxygen and tissue response. For understanding the impact of the fractionated regime definite further investigations are necessary. The observed early apoptosis support the importance of the first illumination in this regime. In future serial observations in time and quantitative analysis may contribute to a better understanding of the apoptosis induced by ALA-PDT in human BCC.

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Chapter 6

Light Fractionation does not Enhance the Efficacy of Methyl 5-Aminolevulinate Mediated Photodynamic Therapy in Normal Mouse Skin

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Abstract

Previous work demonstrated that fractionated illumination using two fractions separated by a dark interval of 2 hours, significantly enhanced the clinical efficacy of photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA). Considering the increasing clinical use of methyl 5-aminolevulinate (MAL) and the expected gain in efficacy by light fractionation we have investigated the response to MAL-PDT using a single and a two-fold illumination scheme and compared that with ALA-PDT. Our results show that fractionated illumination does not enhance the efficacy of PDT using MAL as it does using ALA despite the comparable fluorescence intensities at the end of the first light fraction and at the start of the second light fraction. Only the initial rate of photobleaching was slightly greater during ALA-PDT although the difference was small. Previously we hypothesized that cells surviving the first fraction are more susceptible to the second fraction. Since this is not true for MAL-PDT our data suggest that the distribution of MAL and ALA in tissues, and therefore the site of PDT induced damage, is an important parameter in the mechanism underlying the 2-fold illumination scheme.

Introduction

Photodynamic therapy employing the administration of porphyrin precursors has been under investigation for over a decade (1). It is now the treatment of choice for superficial non-melanoma skin cancer (2) and an experimental therapy for a number of other conditions (3,4). Recently we have shown that light fractionation using a 2-hours dark interval enhances the response of superficial basal cell carcinoma (sBCC) to ALA-PDT significantly (5,6). The increased effectiveness of this type of illumination scheme was first shown in a series of pre-clinical studies (7-10). Initially the fluence was split in two equal light fractions. In a further series of pre-clinical studies using the hairless mouse model we optimised the illumination scheme (10-12). The efficacy of the two-fold illumination scheme increases when the fluence of the first light fraction is relatively low compared to the second light fraction. These data guided us to the design of two clinical studies. The first, a pilot study used a non-optimised two-fold illumination scheme with equal light fractions (5). While the long-term response data in this study showed that complete response rate (CR) remains high compared to CR in the literature, we found no significant difference in CR 1 year after PDT. The second, large-scale randomised comparative study using the optimised illumination scheme showed a significant increase in CR of sBCC at one year compared to ALA-PDT in a single light fraction (6). A number of alternative approaches targeted at enhancing the efficacy in ALA-PDT have been investigated. These have focussed on improving the uptake of ALA and/or the accumulation of protoporphyrin IX (PpIX) using penetration enhancers, iontophoresis, iron chelators (13-17). An important development in this area has been the use of other porphyrin precursors that are derivatives of ALA (18-21).The use of methyl 5-aminolevulinate (MAL/Metvix®) has been studied extensively both in-vitro and clinically. MAL based PDT is now an approved treatment modality for actinic keratosis (AK) in the USA and for non-melanoma skin cancers, such as sBCC and Bowens disease, in Europe and Australia. The recommended treatment protocol involves the topical application of MAL cream (160 mg/g) for 3 hours followed by a single red light illumination and a repetition of this treatment one week later (22).Considering the increasing clinical use of MAL and the expected gain in efficacy by light fractionation we have investigated the response of normal hairless mouse skin to MAL-PDT using a single and a two-fold illumination scheme. Although every two-fold illumination scheme ever investigated using ALA resulted in more damage compared to a single

illumination scheme we chose to investigate the most effective scheme (11,12). We compare these data to our earlier published data on ALA-PDT using the same illumination schemes (12).

Experimental

Animal model

Female inbred albino hairless mice (SKH1 HR, Charles River, Someren, The Netherlands), aged between 8 and 10 weeks, were included in this study. Prior to treatment animals were fed on a chlorophyll free diet (Hope Farms b.v., Woerden, The Netherlands) for a minimum of two weeks in order to remove the autofluorescence emission from mouse skin centered on 675 nm attributed to pheophorbide-a. The animal experiments committee of the Erasmus University Medical Centre approved the experimental protocol.

Chemicals

The ALA cream was freshly prepared as described previously (37). Twenty percent 5-aminolevulinic acid (ALA, Medac, Hamburg, Germany) dissolved in 3% carboxymethylcellulose in water. To prevent skin irritation, the ALA solution was prepared to approximately pH 4 by the addition NaOH (2 M). MAL (Metvix®, 160 mg/g, Galderma, Freiburg, Germany) or ALA was topically applied to a 7 mm diameter area on the dorsal skin and covered with a thin layer of gauze. A polythene dressing (Tegaderm, 3M, The Netherlands) was used to occlude the area for 4 hours prior to treatment. Before application of cream animals received anaesthesia (Hypnorm; fluanisol/fentanyl mixture, Janssen Pharmaceutics, Belgium or Ketamine; Alfasan Woerden, The Netherlands and Diazepam, Centrafarm b.v., Etten-Leur, The Netherlands) to alleviate possible anxiety caused by the dressing.

Experimental Design

The visual and histological response to MAL-PDT using either a single or a two-fold illumination was investigated in two separate series of experiments using the normal mouse model. We have previously shown that visual response data obtained

in this model correlates well with the visual response of UVB induced tumour and tumour growth delay (4,5). We have also shown that optimisation of response in normal mouse skin can lead to the design of clinically relevant fractionation schemes (1,2). In the first series the visual skin damage in the first 7 days after MAL-PDT was investigated (n=6, in each) and compared to that after ALA-PDT using data obtained previously (12). A power analysis showed that this number of animals would be sufficient to resolve a difference in response to the single and two-fold illumination similar to that observed after ALA-PDT. In a second series of experiments the histological damage after PDT with ALA and MAL was investigated. In total 14 groups of mice (n=5-6) were treated. Two groups served as ALA and MAL dark controls in which skin samples were collected at the end of the application period. In the 12 remaining groups skin samples were collected at 2.5, 24 or 48 hours after the end of illumination. In both series the PpIX fluorescence kinetics were recorded during illumination.

PDT Light Delivery and Fluorescence Detection

After 4 hours of topical application with ALA or MAL the skin was illuminated using either a single light fraction of 100 J cm^{-2} at 50 mW cm^{-2} or a two-fold illumination scheme with 5 + 95 J cm^{-2} at 50 mW cm^{-2} separated by a 2 hours dark interval. The experimental set-up used for the PDT illumination and fluorescence detection was based on that described previously (29). The 514 nm output from the argon ion laser was focussed on a 7 mm diameter spot of homogeneous profile on the skin of the mouse. The fluorescence emission from the illuminated area was focussed through a system of lenses onto a CCD camera (ORCA-ER, Hamamatsu, Japan). Excitation light was filtered using a dichroic mirror (535 nm, Omega Optical, Barttleboro, US) and a 635 nm interference filter (Melles Griot, Didam, The Netherlands). Fluorescence images were collected before pre-cursor application, during application and during illumination using the therapeutic light without interruption of the illumination using different light fluences and different integration times. Before each measurement a fluorescence standard was recorded to correct for these differences. The fluence used for each fluorescence measurements before and during the application period was approximately 0.005 J cm^{-2} at 0.5 mW cm^{-2}. Mice were anaesthetized with a combination of 2 % Ethrane or Isoflurane (Abbott, Amstelveen, The Netherlands) oxygen and N_2O for each illumination or fluorescence measurement.

Visual skin Damage

The biological damage to the irradiated area was assessed daily by two independent observers (HSB and APH) blinded from the treatments using the visual skin damage scoring system described previously (33). No change in skin colour was scored as 0. Scores 1 to 3 were used for increasing discoloration of the skin, 1 meaning minimal redness and 3 meaning severe redness. Thin crust formation was scored as 4 and thick crust formation was scored as 5. Damage was observed to be inhomogeneous in a number of treatments. Photographs were taken regularly in order to determine the degree and distribution of damage. The damage score of the total illuminated area in one animal at one time point was calculated by scoring areas according to the degree of damage related to the contribution to the total illuminated area. The visual skin damage of a single mouse was quantified by calculating the area under the curve of skin damage when plotted against time, over the first 7 days resulting in the integrated damage score.

PpIX fluorescence imaging and data analysis

Fluorescence images were recorded before application of the precursor (autofluorescence), during the application period at 1 and 2 hours and during illumination (every 10 seconds). The mean gray scale value was calculated over a large field at the centre of the illuminated area (approximately 65%). To calculate the absolute PpIX fluorescence the background values and the individual autofluorescence were subtracted. A series of measurements during an illumination was normalised to the first measurement of that illumination. The rate of photobleaching over the first 5 J cm^{-2} of each illumination was determined as the slope of the reciprocal of the normalised PpIX fluorescence using linear regression fitting (30). In total 79 mice were illuminated. Data of 16 mice were excluded from analysis due to technical reasons like computer storage failures or focussing and movement problems, resulting in n=12 for the two ALA-PDT groups and n=20 and 19 for the MAL-PDT groups treated according to a single or a two-fold illumination scheme respectively.

Histology

A pathologist (KH) blinded from the treatments scored the histological response by analysing the H&E stained sections of the obtained skin samples. The response of the different layers of the skin was scored separately. We distinguished the epidermis, the upper dermis, the lower dermis and muscle. Tissue damage was observed and scored according to 4 categories: ++ for coagulative necrosis (eosinophilia, loss of structure and undetectable nuclei), + for karyorrhexis (eosinophilia, loss of structure but (fragmented) nuclei could still be recognised) +- for pyknosis (nuclei were shrunk and showed basophilia) and - for no visual damage. The most severe damage observed was scored. Epidermal thickening (hyperplasia) and demarcation of dead tissue by dense polymorphonuclear infiltrates/crust formation were signs of sub-lethal and lethal cellular damage, respectively. Expected late effects are loss of hair follicles and sebaceous glands and dermal fibrosis.

Statistics

The significance of differences in PDT damage and rate of photobleaching was determined using the student-t test and $P<0.05$ was considered significant. Results are shown as mean ± standard deviation.

Results

Response to PDT

The mean visual skin damage scored over the first 7 days post MAL-PDT with a single or a two-fold illumination scheme is plotted in figure 1.

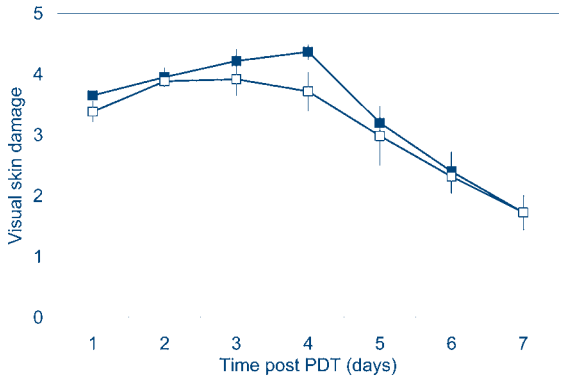

Figure 1: The visual skin damage scores for the first 7 days after MAL-PDT. All groups were illuminated with 50 mW cm^{-2} to a cumulative light fluence of 100 J cm^{-2} delivered in a single fraction (■) or in two fractions (5+95 J cm^{-2} with a 2 hours dark interval) (□). Mean ± SD.

Thin crusts were formed already at day one after MAL-PDT using a single light fraction that developed into thick crusts over time. These crusts became detached at day 4. Light fractionation using the two-fold illumination scheme didn't show a significantly different response. The skin damage at day 1 was less pronounced, crusts were formed in all animals but became detached between day 3 to 5.

The mean of the integrated visual skin damage scores over day 1-7 was 22.6 ± 1.2 and 21.1 ± 3.1 for the single and the two-fold illumination scheme respectively. The use of a two-fold illumination scheme after MAL application did not result in significantly more PDT induced visual damage compared to a single illumination scheme (P=0.29) in contrast to the results previously obtained with ALA-PDT (8).

In general, the histological observations were in agreement with the visual skin damage scores; after a single illumination MAL-PDT resulted in more damage compared to ALA and the increase in damage after a two-fold illumination was not seen using MAL. At 2.5 hours after the end of PDT all groups showed a similar picture of no damage or only slight pycnosis in the epidermal layer. Thereafter

marked differences occurred. While with ALA both a single and a fractionated illumination resulted in a variable response that slightly increased from 24 to 48 hours, MAL-PDT showed a deeper damage in more animals for both illumination and both time points. Figure 2 shows the mean histological response score for each skin layer at each time point for both ALA and MAL. Note that the most severe damage observed in the tissue layer is scored and that we have not compensated for differences in spatial distribution of damage as we have done in the visual skin damage scoring system. Non-illuminated controls showed no damage.

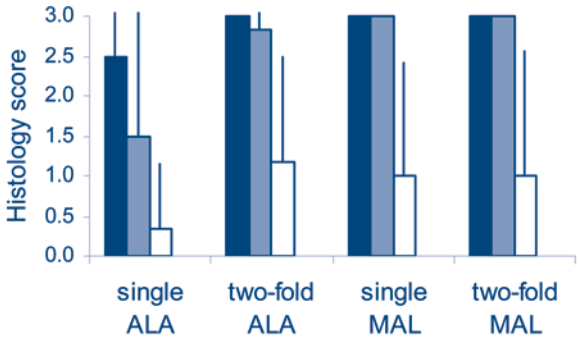

Figure 2: The mean PDT induced damage scored using histology at 24 hours ALA or MAL-PDT. All groups were illuminated with 50 mW cm^{-2} to a cumulative light fluence of 100 J cm^{-2} delivered in a single fraction or in two fractions (5+95 J cm^{-2} with a 2 hours dark interval). The most severe damage was scored in each layer of the skin separately; epidermis (black bar), upper dermis (gray bar) and lower dermis (white bar). Mean ± SD.

Fluorescence Intensity during MAL and ALA Application

The mean PpIX fluorescence kinetics after topical MAL or ALA application and during illumination are shown in figure 3. An insert shows the kinetics during the first 10 J cm^{-2} for clarity. The autofluorescence of normal mouse skin was 39.3 ± 9.7 counts. The PpIX fluorescence 4 hours after the application of ALA or MAL was not significantly different (1107 ± 358 or 1021 ± 408 counts respectively, P=0.40). The relative fluorescence intensity during the application period at 1 and 2 hours was higher using ALA compared to MAL (15 ± 2% and 10 ± 6% at 1 h and 47 ± 11% and 32 ± 13% at 2 h respectively, p=0.17 and 0.22).

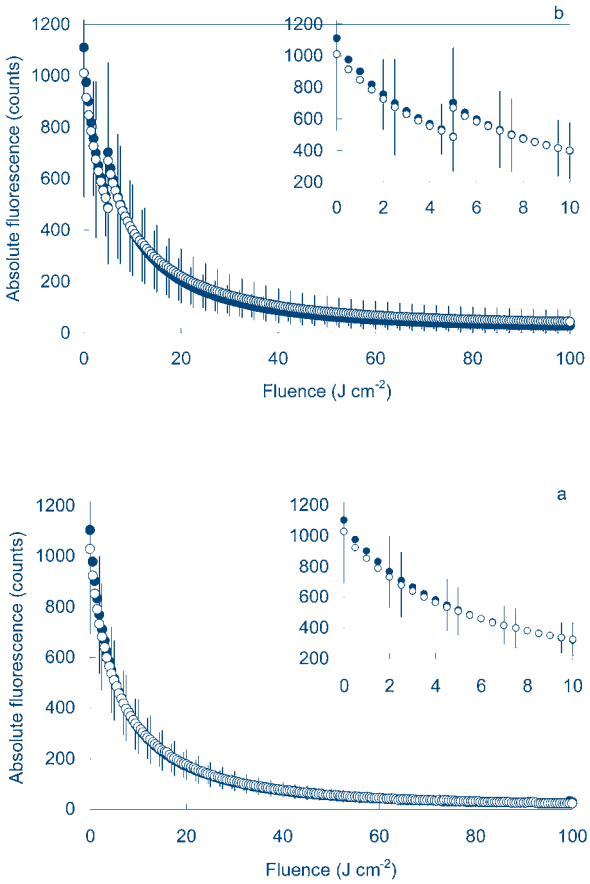

Figure 3: The mean PpIX fluorescence during illumination with 50 mW cm^{-2} to 100 J cm^{-2} delivered in (a) a single fraction or in (b) two fractions (5+95 J cm-2 with a 2 hours dark interval) after topical ALA (●) or MAL (○) application (n=12 for both ALA groups and 20 and 19 for the MAL groups respectively). The results over the first 10 J cm^{-2} are shown in the inserts for clarity. Mean ± SD.

Fluorescence Kinetics during MAL and ALA-PDT

There was no significant difference in the extent of photobleaching during the first illumination using ALA or MAL (46.8 ± 3.3 % and 50.1 ± 5.3% respectively, *P*=0.06). Furthermore, we saw no significant difference in the amount of PpIX re-synthesis during the dark interval between the two light fractions (62.1 ± 14.0 % and 71.3 ± 24.6% respectively, *P*=0.25). The photobleaching kinetics during illumination followed a second order decay for both porphyrin precursors. This is illustrated by the linearity of the reciprocal of the normalized PpIX fluorescence, as

a function of light fluence. Figure 4 shows the kinetics of photobleaching over the first 5 J cm^{-2} for each light fraction.

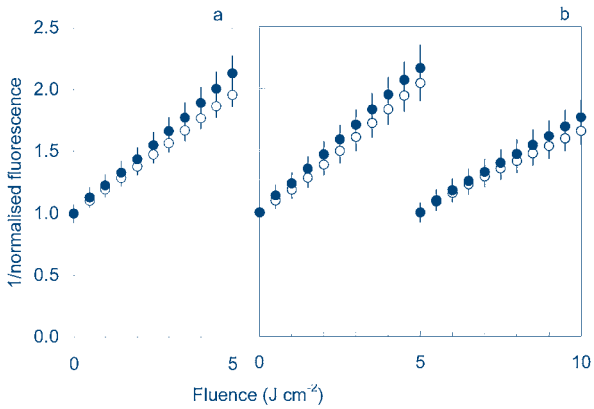

Figure 4: The weighted mean reciprocal of the normalised PpIX fluorescence during the first 5 J cm^{-2} of each light fraction of illumination with 50 mW cm^{-2} to 100 J cm^{-2} delivered in (a) a single fraction or in (b) two fractions (5+95 J cm^{-2} with a 2 hours dark interval) after topical ALA (●) or MAL (○) application (n=12 for both ALA groups and 20 and 19 for the MAL groups respectively). Mean ± SD

Figure 5 shows the variation between animals in the calculated initial rate of photobleaching for each light fraction. Combining photobleaching data from the first 5 J cm^{-2} of the single illumination scheme and the first light fraction of the two-fold illumination, PpIX fluorescence bleached at a greater rate following the application of ALA compared to MAL. While this difference is small it is significant ($P = 0.005$; n=39 for MAL-PDT and n=24 for ALA-PDT). The rate of PpIX photobleaching during the second light fraction of the two-fold illumination was not significantly different using ALA or MAL although for both PpIX precursors it was significantly lower compared to the first light fraction.

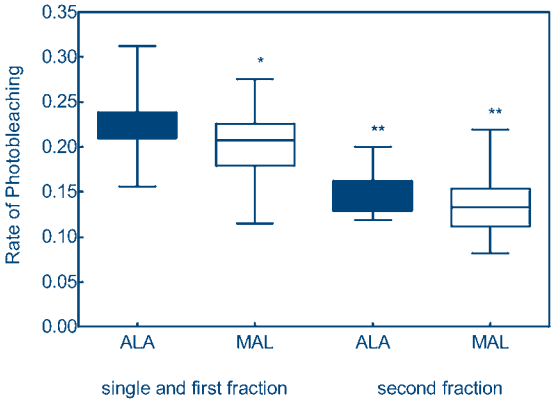

Figure 5: A box & whisker plot of the rate of photobleaching during the first 5 J cm^{-2} of each illumination after topical ALA or MAL application. * significant difference in rate of photobleaching with P=0.005 between MAL and ALA. ** significant difference in rate of photobleaching with P< 0.001 between the first and the second light fraction of the two-fold illumination.

Discussion

Contrary to our expectations, we have found significant differences in the response of normal mouse skin to MAL-PDT compared to ALA-PDT (12). We have shown that there is significantly more visual skin damage following MAL-PDT compared to ALA (12) using a single light fraction although the difference was small and may not be clinically relevant. More importantly, we have also shown that light fractionation does not enhance efficacy in MAL-PDT in the way it does using ALA. Histological examination 24 and 48 h after therapy supports these findings. While the differences in response to PDT using ALA and other pro-drugs of ALA is an interesting subject for investigation they may also expand our understanding of the mechanism underlying the response of tissues to PDT using the two-fold illumination scheme. Numerous in-vitro studies have compared the uptake of ALA and its derivatives, and investigated porphyrin synthesis and cell survival (23,24). However relatively few in-vivo pre-clinical studies have compared the pharmacokinetics of PpIX synthesis (25) and importantly none, to our knowledge, have investigated the response to PDT. Only two clinical studies have compared the clinical response of AK (26) and BCC (27) to ALA and MAL-PDT. Both studies found no difference in clinical response to PDT with ALA and MAL although they were probably insufficiently powered to detect small differences in response of the magnitude that we have observed in the present study.

The pharmacokinetics of PpIX fluorescence in normal mouse skin during the 4-h application period is similar for both porphyrin pre-cursors. At 1, 2 and 4 hours during the application period the fluorescence intensity is not significantly different for animals receiving MAL or ALA. This is in agreement with the results reported by Moan who found similar PpIX fluorescence intensities and concentrations in mouse skin after 1 and 5 hours topical ALA or MAL application (25). We have chosen a 4-h application period for both precursors. While this is longer than the 3 hours that is normally used clinically,Juzenas et al (28) have shown that there is no clear difference in PpIX fluorescence intensity using an application time of 3 or 4 hours. That equal PpIX fluorescence intensities are found immediately prior to the start of the first light fraction is advantageous since large variations in initial PpIX concentration can influence visual skin response for standardised illumination conditions (29).

The kinetics of PpIX fluorescence during the two-fold illumination scheme show that we do not observe significant differences in the extent of photobleaching after each light fraction between ALA and MAL. The kinetics of PpIX fluorescence during MAL-PDT are similar to that published by Moan et al. (30) Also we observed similar amounts of PpIX re-synthesis in the dark interval between light fractions for both ALA and MAL. Therefore the total amount of PpIX utilized during the 2-fold illumination is not significantly different for MAL and ALA. The fact that these observations are accompanied by a dramatically different PDT response adds weight to our previous conclusions that the total amount of PpIX re-synthesized during the dark interval is not involved in the mechanism of action behind the response of tissues to the two-fold illumination scheme (11,31). We have previously shown that the PDT dose delivered during the first light fraction is a critical parameter that can strongly influence the effectiveness of the 2-fold illumination scheme (10,11). Furthermore we have shown that the rate of PpIX photobleaching can be used as an indirect measure of the amount of singlet oxygen produced during PDT and can be used to report PDT dose and correlates with response (29, 32-34). Recently direct evidence for the relationship between the generation of singlet oxygen during PDT and visual skin response in normal mouse skin has been reported (35). Monitoring photobleaching during fractionated ALA-PDT gives an important indication of the PDT dose delivered. A faster rate of photobleaching correlates with a higher PDT dose. In the present study we observe that the rate of photobleaching during the first light fraction of ALA-PDT is slightly but significantly greater than that of MAL-PDT. According to our current understanding of the magnitude of the PDT dose

delivered during the first light fraction on the response of tissues to the 2-fold illumination scheme, this difference in the rate of photobleaching is small and does not explain the lack of an increase in efficacy in MAL-PDT. The rate and extent of PpIX photobleaching during the first light fraction of MAL-PDT is well within the margins of what is considered optimal using ALA (11).

An important difference between ALA and MAL, or other ester derivatives of ALA, is their tissue distribution due to their different biophysical and biochemical characteristics. The cellular uptake of MAL is different from that of ALA (36). While the vehicle in which the pre-cursors are dissolved might also effect the distribution this seems less important since Moan et al (25) used the same vehicle for both MAL and ALA and still showed a different distribution. The authors detected PpIX fluorescence at distant sites after topical ALA application whereas it remained within the application site after MAL application. From this observation they concluded that ALA is systemically distributed after topical application whereas MAL is not. The consequences of these effects for the distribution of ALA or MAL and therefore PpIX within the treatment volume are unknown. It is possible that there is a different distribution of PpIX following the application of ALA and MAL and this has an important effect on the response of tissues to PDT. The present study is limited by the fact that normal mouse skin is relatively thin (approximately 50 μm) and layered and that the PpIX fluorescence is monitored from the surface of the skin. The microscopic distribution of PpIX fluorescence within mouse skin has not been systematically investigated. Besides the difference in (depth) distribution of MAL or ALA within the tissue also other factors may play a role. The microenvironment of a cell may be different when it is loaded with MAL or ALA due to the differences in molecular structure and processes involved in the de-esterification of MAL. How these factors effect the PDT response in mouse skin is unknown but they have not been found to be important factors in vitro (23, 24).A different distribution of MAL and therefore PpIX in the treatment volume leads to a different site of PDT damage and could explain the difference in visual damage following ALA and MAL-PDT using the single illumination scheme. The small but significantly lower initial rate of PpIX photobleaching during MAL-PDT (figure 5, see above) may be a consequence of a different microenvironment due to a different localization of PpIX. We acknowledge that superficial photobleaching measurements are probably not a particularly sensitive method of determining differences in photosensitiser localization. It is important to recognize that it is necessary to perform a first light

fraction followed by a two-hour interval to observe a significant increase in response using ALA (12). Previously we have hypothesized that cells surviving the first light fraction are more susceptible to the second light fraction (12). Since this is not true for MAL-PDT the site of PDT induced damage should be considered as an important parameter in the mechanism underlying the 2-fold illumination scheme. Apparently the site of PDT induced damage combined with the two-hours dark interval between light fractions is important for the increased efficacy observed using light fractionation during ALA-PDT.

We have previously shown that pre-clinical results obtained in normal mouse skin using ALA-PDT can be successfully translated to a clinical treatment protocol for sBCC that results in a significantly improved CR (6). The results of the present study imply that simply combining MAL-PDT and the most effective two-fold illumination scheme, determined for ALA, in the clinic would not enhance the efficacy of MAL-PDT using a single light fraction. It is possible that the illumination parameters used in the present study are not optimal for MAL. However, it is important to note that light fractionation in ALA-PDT always enhances the response of tissue compared to a single illumination. We note that more pre-clinical research on the mechanism behind the increased effectiveness of the two-fold illumination using ALA and the mechanism of PDT induced damage using MAL is needed to confirm this assumption.

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Chapter 7

Quality of clinical practice guidelines in dermatological oncology

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Abstract

Background: Clinical practice guidelines are increasingly used. To determine the quality of guidelines the Appraisal of Guidelines and Research and Evaluation (AGREE) instrument was developed and introduced in 2001. The AGREE instrument consists of 23 criteria, grouped in six domains.

Objective: Assessment of quality of evidence-based guidelines in dermatological oncological care according to the AGREE instrument.

Methods: We searched MEDLINE, PubMed, EMBASE and Cochrane literature and relevant websites of guidelines development programmes and the national dermatological society to identify evidence-based dermatological guidelines especially in the treatment of basal cell carcinoma, squamous cell carcinoma and melanoma. Twenty guidelines, published between 1990 and 2005, were appraised according to the AGREE instrument by three authors. Standardized domain scores were calculated as advised by AGREE. We compared guidelines published before 2002 with those published later.

Results: Domain scores in the domains Scope & Purpose and Clarity were scored best. Applicability and Editorial Independence were scored worse (see Table 1). In time a weak trend towards better guidelines was seen. This trend can be attributed to better scores in the domains Search Strategy and Level of Evidence which are closely related to evidence-based medicine. The increase in score is due to more explicitly mentioning the search strategy, possible conflict of interest and involvement of different specialties in development of the guideline. Using the Mann–Whitney test to compare guidelines published before the AGREE and afterwards only a statistically significant better score was found for the domain Clarity ($P < 0.05$; Table 2).

Conclusions: Guidelines in dermatological oncological care are of reasonable quality according to the AGREE instrument. The domains in the AGREE instrument concern especially the methods of development of guidelines. The score according to AGREE can be improved by explicitly mentioning the different items. As clinical guidelines are regarded to be an important instrument to improve quality of care, improvements are needed.

Introduction

Clinical practice guidelines are increasingly used throughout the world to improve the quality of patient care.(1) They are expected to facilitate more consistent, effective and efficient medical practice and improve health outcomes. The quality of clinical practice guidelines is varying. Underlying principles of evidence-based medicine seems to be clear, but in daily practice it may be hard to judge quality of the methodology of published guidelines and their clinical content. Several studies have suggested that many guidelines are of poor quality. (2–5) The research community recognized the need for internationally accepted criteria for guidelines and in 1998 the Appraisal of Guidelines and Research and Evaluation (AGREE) project was established. Researchers from 13 countries developed the AGREE instrument.

This is a validated generic instrument for the appraisal of guidelines applicable to any disease.(6)

The instrument, published in 2001, provides a tool consisting of 23 criteria, grouped in six domains (Scope & Purpose, Stakeholder Involvement, Methodology, Clarity and Presentation, Applicability, and Editorial Independence; see Appendix 1, www.agreecollaborationgroup.org). This instrument is designed to assess the process of guidelines development and how well this process has been reported. In this article, we present a quality assessment of clinical practice guidelines on dermato-oncological care. We assessed the quality of guidelines on the treatment of basal cell carcinoma, squamous cell carcinoma and melanoma.

Methods

*Selection of guidelin*es

The authors conducted a MEDLINE, PubMed, EMBASE and Cochrane literature search of guidelines from 1990 to 2005 using search terms alone and/or in combination: skin disease, practice guidelines, healthcare planning,evidence-based medicine, meta-analysis, consensus development. In addition, a search was performed using the search engine provided by the websites of guideline development programmes and national dermatological societies. The search provided 200 guidelines. By narrowing the search to guidelines concerning the three most common forms of skin cancer – melanoma, squamous cell carcinoma and basal cell carcinoma – 59

guidelines remained. Search results that were duplicates of the same guidelines, published before 1990, background documents were removed. Twenty guidelines remained and were appraised according to the criteria of the AGREE instrument (see Appendix 2). Two versions of the Dutch melanoma guideline were appraised, one version published in 1997 and the updated version published in 2005. A comparison of two groups of guidelines was performed: those published before 2002 reflecting the guidelines published before the AGREE instrument and those published afterwards.

Appraisers

Three authors (EdH, HdV, WSvR) assessed the quality according to the AGREE instrument. HdV and WSvR are residents in dermatology. EdH is a dermatologist.

Analysis

Standardized domain scores are calculated as advised by AGREE. A standardized score above 60% is concordant with a positive agreement of the involved criteria. All appraisers scored individually. The definitive score was noted in consensus. To compare differences in scoring between guidelines published before the introduction of the AGREE instrument in 2001 and after, the guidelines published before 2002 and afterwards were statistically compared by using the non-parametric Mann–Whitney *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

There was a marked difference in scoring between the six domains in all 25 guidelines as is shown in Table 1. A standardized domain score above 60% is considered to be good and is marked in the table.

Table 1: Standardized domain scores

author	country	Year of publ.	Scope &Purpose	Stakeholders	Rigour	Clarity	Applicability	Editorial indepen-dence
Drake	USA	1992	28	21	5	13	0	0
Drake	USA	1993	***72***	25	14	25	0	0
Drake	USA	1995	***67***	25	5	25	0	0
De Ruiter	NL	1997	33	21	29	***66***	11	3
Cox	UK	1999	***67***	33	24	46	6	
Telfer	UK	1999	***89***	29	43	58	28	0
Reeve	AUS	1999	***100***	***100***	***88***	***71***	44	0
Negrier	FR	2000	***72***	46	48	***66***	0	50
Dummer	Swiss	2001	***83***	***63***	36	***63***	0	8
Cook	USA	2001	***83***	25	***67***	58	11	50
Sober	USA	2001	***100***	38	***67***	***67***	0	33
Sober	USA	2001	***100***	33	55	42	0	0
Motley	UK	2002	***83***	25	45	58	0	33
Motley	UK	2002	***89***	38	40	***67***	0	0
Roberts	UK	2002	***78***	33	45	58	0	0
Roberts	UK	2002	***100***	50	29	***71***	***61***	0
Marks	AUS	2002	***100***	***100***	57	***71***	50	0
Beljaards	NL	2003	***78***	54	***88***	***67***	44	0
Doherty	Scotland	2004	***89***	***88***	***69***	***88***	39	***75***
Rademaker	NZ	2004	11	25	17	50	0	0
Houghton	USA	2004	56	33	52	***67***	44	0
Quirt	Canada	2004	***100***	***75***	***93***	71	56	58
Miller	USA	2004	33	4	21	38	0	0
De Ruiter	NL	2005	***62***	54	***95***	***79***	33	33

The domains Scope & Purpose and Clarity were scored best. Eighteen guidelines scored above 60% in the domain Scope & Purpose and 12 guidelines scored above 60% in the domain Clarity. The domain Editorial Independence was scored worse. Only one guideline scored above 60%. Guidelines are listed in chronological order. The total score of the updated version of the Dutch melanoma guideline published in 2005 is higher than that of the first version published in 1997. The median score of guidelines published before and after 2002 only the domain Clarity shows a statistically significant different score ($P < 0.05$; Table 2).

Table 2: Statistics

Guidelines before AGREE, n=12	median	Percentile<	>
Scope & purpose	***77.5***	<67.0	97.3>
Stakeholders	31.0	<25.0	44>
Rigour	39.5	<16.5	64.0>
Clarity	58	<29.3	66.0>
Applicability	0	<0	11>
Editorial independence	4	<0	33>

Median *standardized domain score of guidelines published before the AGREE instrument (<2002).*

Guidelines after AGREE, n=11	median	Percentile<	>
Scope & purpose	***83***	<56.0	100.0>
Stakeholders	38	<25.0	75.0>
Rigour	45	<29.0	69.0>
Clarity	***67***	<58.0	71.0>
Applicability	39	<0	50.0>
Editorial independence	0	<0	0>

Median *standardized domain score of guidelines published after the publication of the AGREE instrument (>2002)*

Domain	P<	
Scope & purpose	0.74	
Stakeholders	0.32	
Rigour	0.35	
Clarity	***0.05***	9.3-14.9
Applicability	0.21	
Editorial independence	0.38	

Guidelines published before AGREE and guidelines published after publication of the AGREE instrument.
Using non parametric t test Mann Whitney

Discussion

Clinical practice guidelines are increasingly used. Several studies suggested that many of them are of poor quality.(2–5) In order to improve the quality of guidelines,

the AGREE instrument was developed and introduced in 2001. This instrument is used for judging and developing guidelines.(7–14) We used the AGREE instrument to appraise 20 guidelines. Those guidelines are of reasonable quality: all but two guidelines had at least one domain scoring above 60% which is considered to be good. In the course of time between 1990 and 2004 more often the methods of searching are mentioned explicitly, combined with a level of evidence. Besides, conflict of interest is mentioned more clearly. The appraisal of the Dutch melanoma guideline published in 1997 compared to its updated version published in 2005 is a good example of improvement in scoring in time. However, we did not demonstrate an overall improvement in guidelines in time. A statistically significant difference between the guidelines published before the introduction of the AGREE instrument and those published afterwards is only seen in the domain Clarity ($P < 0.05$). Our opinion is that guidelines of higher quality are developed by using the 23 criteria of AGREE. However, describing the 23 criteria in the guideline results in a large document and guidelines need to be clear and short for easy use. For that reason a short overview on a separated card is added to guidelines in the Netherlands. Useful additional information-related literature and links can be discussed at length in a background document.

Evidence-based medicine and AGREE

Evidence-based medicine is a methodology for evaluating the validity of research in clinical medicine and applying the results to the care of individual patients. Evidence is gathered through systematic review of literature, and is critically appraised. The results are then integrated with physician/patient decision-making. Evidence-based medicine (EBM) uses a simple scale to assign a level to evidence. Systematic reviews that summarize randomized controlled trials are assigned the highest level of evidence. Expert opinions and 'personal communication' are assigned the lowest level of evidence. The AGREE instrument is based on the principles of evidence-based medicine. It originates from an international collaboration of researchers and policymakers, who work together to improve the quality and effectiveness of clinical practice guidelines by establishing a shared framework for their development, reporting and assessment. According to EBM and AGREE, guideline development requires a clearly defined and dated literature search and defined levels of evidence. Mentioning search strategies makes it easy for users to weigh the quality of a guideline. Appraising the actuality of the guideline and suggestions for updating

are underexposed aspects in the AGREE instrument. By mentioning those aspects more explicitly in guidelines an updating can be made by the user himself or herself. In general the lifespan of a guideline is regarded to be 3 years.(15) In the appraised guidelines a limited lifespan is recognized, but only in one-third of them information about updating is given. Guidelines based on substantial high level of evidence may be more robust over time than those based substantially on experts views.(16) On the other hand, using EBM and levels of evidence introduce a risk of overweighing randomized controlled studies. Experts' opinions and views of stakeholders in the field represent an important part of evidence especially because of the longstanding empirical base of medical science. (17,18) We suggest to use the most complete literature to assign levels of evidence in guidelines. The actuality and an estimated lifespan should also be included.

Patients opinion and AGREE

In guidelines concerning oncological treatment, opinions and preferences of patients should be included. Especially important in situations in which 'quality of life' plays an important role, the patient's opinion is crucial. Subjects such as morbidity, side-effects, cosmetic outcome and time consumption can be an important aspect for patients.(19) In the appraised guidelines these aspects are not (clearly) included. Patients' instructions, information brochures and other supporting material are often not a part of the guideline. In some guidelines, addresses or Internet links of supporting societies are provided for general support or support in a special field, for example genetic counseling. Sufficient information and communication is nowadays an important requirement in general healthcare.Quality of guidelines can have an influence on the quality of care. Quality of guidelines should not only be regarded in the development phase but also in the practical phase.(20) Furthermore, quality of care needs acceptance, implementation and evaluation in a circular way, finally leading to improvement.(21) Acceptance and implementation is also depending on the impact on organizations and patient outcomes as financial consequences and recognition of existing barriers. (22–24) Only few guidelines mention these factors.

Conclusion

In this study, we used the standardized domain scores of AGREE to appraise guidelines in dermato-oncological care in which we conclude most guidelines to be of reasonable quality but improvements are possible and needed as clinical guidelines are regarded to be an important instrument to improve quality of care. In course of time, trends towards better developed or at least better documented development of guidelines is seen. Although only in one domain (Clarity) a statistically significant difference is seen between guidelines published before the AGREE instrument and after the AGREE instrument. This may be seen as an illustration of the limitations of the AGREE instrument. We believe that the AGREE instrument is especially of value for those involved in guidelines development more than to be an instrument to evaluate quality of guidelines. The 23 criteria can serve as a recipe to prepare a complete guideline reflecting EBM and shared opinions for all participants and possible users.

Appendix 1: AGREE domain structure

Scope and purpose (item 1-3) is concerned with the overall aim of the guideline, the specific clinical questions and the target population.

1. The overall objective(s) of the guideline is (are) specifically described
2. The clinical question(s) covered by the guideline is (are) specifically described
3. The patients to whom the guideline is meant to apply are specifically described

Stakeholder involvement (items 4-7) focuses on the extent to which the guideline represents the views of its intended users.

4. The guideline development group includes individuals from all the relevant professional groups
5. The patients' views and preferences have been sought
6. The target users of the guideline are clearly defined
7. The guideline has been piloted among end users

Rigour of development (items 8-14) relates to the process used to gather and synthesise the evidence , the methods to formulate the recommendations and to update them.

8. The systematic methods were used to search for evidence

9. The criteria for selecting the evidence are clearly described
10. The methods used for formulating the recommendations are clearly described
11. The health benefits, side effects and risks have been considered in formulating the recommendations
12. There is an explicit link between the recommendations and the supporting evidence
13. The guideline has been externally reviewed by experts prior to its publication
14. A procedure for updating the guideline is provided Clarity and presentation (items 15-18) deals with the language and format of the guideline.
15. The recommendations are specific and unambiguous
16. The different options for management of the condition are clearly presented
17. Key recommendations are easily identifiable
18. The guideline is supported with tools for application

Applicability (item19-21) pertains to the likely organizational, behavioural and cost implications of applying the guideline.

19. The potential organisational barriers in applying the recommendations have been discussed
20. The potential cost implications of applying the recommendations have been considered
21. The guideline presents key review criteria for monitoring and/or audit purposes

Editorial independence (item 22-23) is concerned with the independence of the recommendations and acknowledgement of possible conflict of interest from the guideline developmentgroup.

22. The guideline is editorially independent from the funding body
23. Conflicts of interest of guideline development members have been recorded

Appendix 2: guidelines authors and references

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16 Doherty et al, SIGN & NCH 2003; Cutaneous melanoma.

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Chapter 8

Fractionated ALA-photodynamic therapy provides additional evidence for the use of PDT for non-melanoma skin cancer

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Abstract

Background: PDT is an accepted treatment for sBCC and Bowens disease. In Rotterdam extensive preclinical research has lead to an optimized two fold illumination scheme for ALA-PDT.

Objective: To provide additional evidence of ALA-PDT for sBCC, BD, nBCC and AK using a two fold illumination scheme after a single application of ALA.

Methods: 552 lesions (430 sBCC, 20 nBCC, 32 BD, 70 AK) were treated with ALA-PDT using a 2-fold illumination scheme. ALA was applied topically for 4 hours. Lesions were treated with two light fractions of 20 and 80 J cm^{-2} separated by a 2 hours dark interval.

Results: After a minimum FU of 12 months, in average FU 2 years an overall CR of 95% was seen for all lesions. For sBCC the CR at two years was 97%, for AK 98%, for BD 84 % and for nBCC 80% . A sub- analysis of the results of lesions larger than 2 cm's showed CR at two years of 89% for all lesions (n=57). Cosmetic outcome was good to excellent in 95% of the treated lesions.

Conclusion: ALA-PDT using a two fold illumination scheme of 20 plus 80 J cm^{-2} separated by a two hours dark interval leads to high CR rates at two years and can be regarded as an evidence based treatment modality for superficial growing non melanoma skin cancer and the (pre)malignant AK.
The Rotterdam fractionated approach should be included in future guidelines.

Introduction

Photodynamic therapy (PDT) is an accepted treatment for non melanoma skin cancer especially superficial growing lesions (1). The use of 5-ALA in a topical preparation was first described by Kennedy and Pottier in 1990 (2). Since then many studies and case series have been published. In 2001 the FDA (Food and Drug Administration) approved the use of topical ALA (Levulan ®) combined with blue light in the treatment of actinic keratosis (AK). The methyl-ester of ALA (MAL, Metvix®) was subsequently introduced and registered for treatment of superficial basal cell carcinoma (sBCC) and AK in 2004 and for treatment of Bowens disease in 2006 in most European countries. Although the mechanism behind PDT using porphyrin precursors is still not completely elucidated, high complete response rates combined with excellent cosmetic outcome have been obtained in many studies. PDT is now included in several clinical guidelines (1, 3). Extensive preclinical research in Rotterdam stimulated clinical participation and clinical trials which resulted in three recently published clinical studies using ALA-PDT with a two fold illumination scheme for the treatment of superficial non melanoma skin cancer (4,5,6). In addition to these research clinical studies PDT is now part of our daily practice in which we utilize light fractionation. This treatment scheme is well tolerated by patients and results in excellent cosmesis. Recently Braathen et al (1) published an international consensus in which they formulated guidelines on the use of PDT. Guidelines are increasingly important in dermatology. As guideline development is an ongoing process, the results achieved in Rotterdam have important implications for future guidelines on PDT.

Here we present an analysis of a subgroup of patients treated in our centre. Our clinical work to date in treating patients with AK, sBCC, nBCC and Bowens disease shows high complete response rates and adds evidence to PDT as a treatment modality for superficial skin cancer.

Patients and Methods

Patients

All patients were diagnosed in our department of Dermatology in Rotterdam. In patients with sBCC 39% of lesions were histological proven, all patients had at

least one primary sBCC diagnosed histologically. All nBCC and Bowens disease lesions were histological proven. For patients with AK, 66% of lesions were histological proven. Our patient population consists of both first-line and secondary dermatological care. All patients are adult Caucasians. The mean age for AK was 62 (range 53-86), for nBCC 59 (range 45-75), for sBCC 58 (range 33-81), for Bowens disease 74 (range 50-90). Sex distribution was in AK 17males, 15 females; in sBCC 53 males, 37 females; in nBCC 12 males, 4 females; in Bowens disease 10 males, 16 females. The average diameter of lesions was 10.6 mm for AK, 10 mm for sBCC, 10.3 mm for nBCC and 16 mm for Bowens disease. The overall range in lesion size was between 3 and 70 mm (see also table 1). Lesions were located at different sites of the body, details are shown in table 1, the most commonly treated lesion was a sBCC on the trunk. We note that the clinical response data from a small number of the patients with sBCC and Bowens disease have been reported previously (5, 6).

Methods

Our methods were similar to those that we have described previously (5). In brief, the topical ALA ointment we used was freshly prepared by our Hospital Pharmacy using 20% ALA (FLUKA, The Netherlands) in Instilagel (Medeco BV, Oud Beijerland, The Netherlands).The ointment was stored in a refrigerator and used within 7 days. Before application of ALA, crusts and scaling were gently removed using a disposable curette. The lesion was covered with a margin of 1 cm and dressed with a semipermeable dressing (Tegaderm 3M, The Netherlands) and light-protecting covering (Aluminum foil). In addition to the topical anaesthetic in the ALA ointment, patients received field block anaesthesia with lidocaine 1% (without epinephrine) if required. Three light sources were used. A 630 nm diode laser (Carl Zeiss, Oberkochen, Germany) was used to provide 630 nm illumination. Two commercially available broadband light sources were also used. The first had a spectral output between 590 and 650 nm (Medeikonos, Gothenburg, Sweden). The second was a light-emitting diode, a light source with a spectral output centered on 633nm with a bandwidth of 20nm (Omnilux, Waldmann, Tiel, The Netherlands). All three light sources were used to illuminate lesions with a margin of at least 5mm at a constant measured fluence rate of 50 $mWcm^{-2}$. Lesions received light fractions of 20 and 80 $J\ cm^{-2}$, 4 and 6 hours after a single application of ALA. During the 2-h dark interval between light fractions, lesions were covered with a light protective dressing.

Results

Analysis of our treated patients in Rotterdam with a minimum follow up of 12 months is shown in table 1.

We treated in total 552 non melanoma skin cancer lesions (AK, Bowens disease, superficial and nodular BCC) with fractionated ALA-PDT. The majority were sBCC's. After a follow up at least 12 months with an average two years (range 12-42) a complete response rate of 95% was observed . Bowens disease lesions that showed partial response were retreated and responded in all cases (follow up 12 months after re-treatment). These data were not included as responders in the present analysis. The response rate for AK was 98%.The response rates for nodular BCC and M Bowen were 80% and 84% respectively.

Table 1: Results of fractionated ALA-PDT at a minimum follow up of 12 months

	AK	sBCC	nBCC	Bowens	Remarks
No lesions	70	430	20	32	
No patients	32	90	16	26	
Histological proven	46 (66%)	167(39%)	20(100%)	32 (100%)	
Localization					
vertex	6	5			
face	25	43	6	9	
arm	14	77	2	10	
leg	9	43	1	8	
trunk	16	262	11	5	
Average size of lesions	10.6mm	10mm	10.3mm	16mm	
Min-max	3-25mm	4-50mm	4-30mm	3-70mm	
Light source used					
Diode laser	2	45	4		
Medeikonos	12	87	4		
Waldmann omnilux	56	298	12	32	
Pain requiring local lidocain	11 (16%)	20(5%)	-	5 (16%)	All AK lesions on vertex
CR	69 (98%)	419(97%)	16(80%)	27 (84%)	Bowen's after second PDT treatment CR 30(94%)
FU average	20 months	23months	23months	20 months	
FU range	12-41 months	12-42 months	12-31 months	12-32 months	
Cosmetic outcome					
fair	4 (6%)	18(4%)	2 (10%)	2(6%)	
good	66	412	18	30	

A subanalysis of the larger lesions with a diameter above 2 cm for all types of lesion is shown in table 2. In total 57 lesions were above 2 centimeters in diameter. A complete response at a follow up of two years of 89% was seen for these large lesions. The complete response rate for AK lesions was 100% at two years. Bowens disease lesions larger than 2 cm responded in 78% and sBCC lesions in 91% at a follow up time of 24 months. The complete response of large nodular lesions was lower, a complete response of 50% after 24 months follow up.

Table 2: Results of fractionated ALA-PDT for AK, Bowens disease, sBCC and nBCC lesions larger than 2 cm with a minimum follow up of 12 months (range 12-40), mean 22 and median 23 months

	AK	M Bowen	sBCC	nBCC	remarks
No lesions	14	9	32	2	
No patients	12	9	20	2	
Localization					
Vertex	3				
Face	2	1	4	1	
Arm	5	1	1	1	
Leg	1	4	4		
Trunk	3	3	23		
Light source used					
Diode laser			5	1	
Medeikonos	1	4	15		
Waldmann omnilux	13	5	12	1	
Pain requiring local lidocain	3	1			
CR	14 (100%)	7(78%)	29(91%)	1(50%)	
Cosmetic outcome					
Fair	2		4	1	
Good	12	9	28	1	

Fractionated ALA-PDT is well tolerated. Patients required only in few lesions local anesthesia during treatment. The cosmetic outcome varies from excellent to good. Fair cosmetic outcome was only seen in 5% of the lesions and was due to (transient) hypo-or hyperpigmentation.

Discussion

The use of ALA-PDT with a fractionated illumination scheme, in which two light fractions are delivered separated by a two-hours dark interval, following a single ALA application, is in our opinion, an evidence based PDT treatment modality. This conclusion is based on clinical response data published in the literature (4,5,6) and the data presented in this article. The fractionated illumination scheme has been designed, based on a range of pre-clinical studies in a variety of pre-clinical models (10). Results of these studies show that fractionated ALA-PDT results in significantly higher responses compared to ALA-PDT performed with a single illumination (11,12). Studies have also shown the importance of the time interval between ALA application and the first light fraction and its relation to the synthesis of PpIX (13). Besides the influence of the fluence (rate) of the individual light fractions (13,14) and importantly the time interval between light fractions has been shown to be critical (15). Enhanced PDT response is achieved if two light fractions are delivered 4 and 6 hours after the application of ALA, separated by a 2 hours dark interval. Dividing the illumination in a small first and a substantially larger second light fraction further enhance the PDT response (12). The mechanism behind this increase in response is under investigation (16) but seems to be closely related to the response of the vascular endothelial cells in-vivo.

Our first (pilot) study was performed using an illumination scheme 45 Jcm^{-2} + 45 Jcm^{-2} separated by a two hours dark interval. A follow up of mean 59 months (range 44-82) with a complete response rate of 84% and excellent cosmetic outcome in 88%.(4) Secondly we did a large comparative study in superficial basal cell carcinoma (n=505) comparing a single illumination (75 Jcm^{-2}) with a fractionated illumination (20+80 Jcm^{-2}) with a two hours dark interval. The results clearly favor fractionated illumination. Complete response rate in the fractionated illuminated lesions was 97%, in the single illuminated group the complete response rate was 89% ($p<0.002$ log rank) (5). We also studied the response of squamous cell carcinoma in situ (Bowens disease) using the same parameters as in the basal cell carcinoma study. In this (pilot) we included 50 patches of Bowens disease. These results were also in favor of fractionated illumination (80%vs 88%) but did not reach statistical significance (possibly due to the small number of lesions included)(6). Nodular BCC do respond, but in all studies (ALA and MAL) this seems to be limited (17,18,19). More research to define criteria according to which lesions are suitable for ALA-

PDT using light fractionation is necessary. Prior curettage and repeated optimized light fractionated ALA-PDT are also possible options. It is important to investigate the maximum depth of PDT damage and the different mechanisms of PDT as direct and indirect damage, necrosis, apoptosis and immunological responses to malignant and surrounding cells including the vasculature. The data we have shown here establish our previous clinical work. Besides they show high response rates for AK. AK is considered as pre-malignant condition which has the potential to become malignant. The need for optimization of the treatment of AK is highlighted by the recent classification of these lesions as carcinoma in situ. (20). The results we present using light fractionated ALA-PDT shows optimization for sBCC, Bowens disease and AK. Changes in application time, illumination time and other parameters will influence the ultimate PDT effect. Changes should be subject of well designed research and not be used as trial and error.

To improve the quality of medical care guidelines are extensively used. Guidelines are expected to facilitate more consistent, effective and efficient medical practice and improve health outcomes. In 1998 Appraisal of Guidelines and Research and Evaluation (AGREE) project was established (7, www.agreecollaborationgroup.org). These criteria can be used to appraise a guideline but can be considered of importance in the developmental phase (8,9). Recently the American Academy of Dermatology (1) published an international consensus in which guidelines are formulated on the use of PDT. This shows clearly the importance of PDT in the treatment of non melanoma skin cancer. As guideline development is an ongoing process we would like to suggest in future to develop an integrated guideline for PDT and the other treatment modalities for non melanoma skin cancer according to the AGREE instrument. Results achieved in Rotterdam should be incorporated.

In Conclusion

PDT is an evidence based treatment modality for superficial growing skin cancer like superficial basal cell carcinoma, Bowens disease and actinic keratosis. A fractionated illumination of 20 + 80 Jcm^{-2} separated by a two hours dark interval after a single application of 20% ALA in treatment of sBCC, Bowens disease and AK-lesions achieve high complete response rates after a two year follow up period. To a lesser extent is this high complete response rate established for the treatment of nBCC

using light fractionated ALA-PDT. Treatment optimization for nodular lesions and/or better defined lesion inclusion criteria are desirable. Light fractionated ALA-PDT should be incorporated in future clinical guidelines for non melanoma skin cancer.

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Chapter 9

Chasing the obvious?

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The Importance of Patient reported Outcomes (PRO)

In the evaluation of therapies, the physician-based clinical model (e.g., physician global assessment, change in affected body surface area, index scores, disease free interval, survival and recurrence rate) alone is no longer sufficient. In addition to traditional methods of evaluating disease, regulatory agencies now require the assessment of the patient reported outcomes (PRO).(1) These outcomes are pivotal in dermatology, because most dermatological diseases are chronic, intermittent, non fatal, very visible, cause no permanent physical damage and their disease activity can not be measured using serological markers. Initially, PRO focused on inflammatory dermatoses, but it now also includes non inflammatory diseases and (benign and malignant) neoplasms, which are associated with to some extent different and specific issues. Because non melanoma skin cancers (NMSC) rarely metastasize, cause few symptoms and treatment is often locally, interest in patients' perspectives on their skin cancers and its treatments has been very recent. However, NMSC *are* cancers, often located on the face, surgery may be associated with substantial fear, functional and cosmetic morbidity, and the risk of subsequent skin cancer is close to 50% making this a chronic disease. These issues are troubling to patients and should be incorporated in the assessment of global disease severity and evaluation of skin cancer therapy.(2) A better understanding and documentation of patients` views is helpful in improving patient-physician interaction, skin cancer care, treatment outcome and the selection of patients in need of additional pre-treatment counseling.

In clinical studies, there are three obvious 'players' that should be evaluated separately (i.e., disease, patient and intervention). The disease is evaluated using traditional clinical methods, the effect of the intervention on patients` lives is captured by health related quality of life (HRQOL) and symptom assessment, and treatment is evaluated by assessing its adverse events and satisfaction with medication or intervention (Figure 1). In addition to physicians who assess clinical measures, patients are more and more actively involved in judging the other aspects of therapy and their opinions as end users are increasingly important. Of these PROs, HRQOL is by far the most commonly used measure. The World Health Organization (WHO) defines QOL as `the individuals` perception of their position in life, in the context of the cultural and value systems in which they live and in relation to their goals, expectations,

standards and concerns`. QOL is multidimensional and includes health and multiple non-medical aspects such as socioeconomic status, marital status, professional career, personality, expectations and religion. In medicine, QOL measures focus on HRQOL that should measure physical, psychological and social health domains both in a subjective and objective manner (3,4).

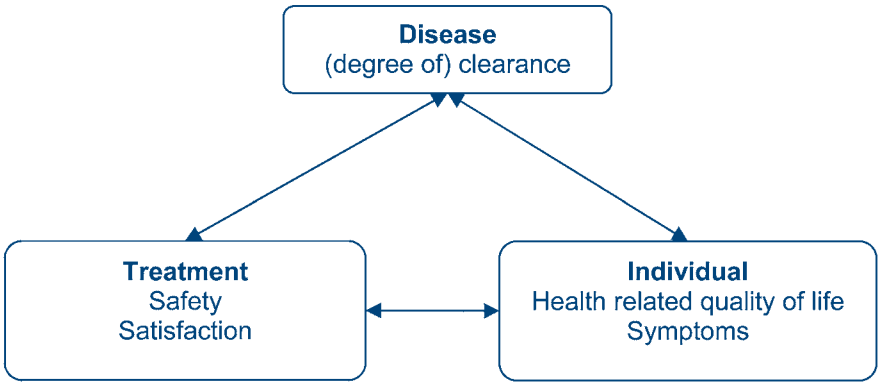

Figure 1: The three key players and corresponding outcomes in the evaluation of a treatment.

HRQOL in Skin Cancer Patients

Of the available generic or dermatology specific HRQOL instruments, the Short Form (SF)-36, Sickness Impact Profile (SIP), Dermatology Life Questionnaire Index (DLQI) and Skindex-16 have been used to assess the impact of BCC on patients' lives and demonstrated a minimal to little impact of this disease.(5-9) This can, in part, be explained by the fact that these instruments lack specificity (i.e., asking about concepts relevant to patients with skin cancer), sensitivity (i.e., detects small impairments) and responsiveness (i.e., detects HRQOL changes after improving/ worsening of health status) in patients with NMSC. These non specific instruments are likely to suffer from 'floor' effects (i.e., substantial proportion of respondents will tick off the lowest response category) and summing scores are likely to be in the lowest quartile of the instruments' range because several pivotal concepts such as susceptibility of skin cancer, worrying about (facial) health problems, appearances (e.g., scars and dysmorphism), fear of recurrence or new skin cancer, and fear of surgery are not assessed (in detail).(9) The skewed distribution of item responses

and total scores to the low levels make it difficult for these tools to differentiate within the range of limited impairment and to detect changes over time. Therefore, skin cancer specific HRQOL questionnaires such as the Skin Cancer Index have been introduced recently, which should be validated before they can be used in conjunction of dermatology specific, cancer specific and/or generic tools.(2,10,11)

Recently, Chren and colleagues have used HRQOL as a primary outcome in a comparative cohort study in the treatment of NMSC. This very interesting study showed that the emotional impact of NMSC was almost three folds compared to that of functioning and symptoms as measured by the Skindex-16.(12) Also, patients treated with surgical excision and Mohs surgery but not electrodessection and coagulation (ED&C) improved in all three HRQOL domains.

Predictors of HRQOL Improvement

In this issue of the *Archives*, the same group investigated the predictors of this observed HRQOL improvement after skin cancer therapy. The objective of this study was to understand why NMSC therapy affects people in different ways and to identify individuals who are at higher risk of not experiencing a therapeutic benefit after a 'successful' therapy. The authors have used a heterogeneous cohort of 663 patients with new basal cell carcinoma (BCC; 77.3%) and squamous cell carcinoma (SCC; 32.7%) who received therapy at a VA or private practice.(12) Of multiple patient, tumor and care characteristics, pre-treatment skin related HRQOL, comorbidity, health status (i.e., SF-12 scores) and being white significantly improved post-treatment Skindex-16 scores after adjusting for confounding factors in a multivariate model. The strongest independent predictor of skin-related HRQOL post-treatment was pre-treatment skin-related HRQOL. This is in accordance with a previous study showing that individuals` pre-operative health beliefs such as worrying, fear and susceptibility determine for a large part the extent to which patients respond to BCC therapy.(2) Therefore, clinicians should gauge patients` perceptions of NMSC and its treatment and inform patients about both before therapy is administered, especially those who express higher levels of worrying and fear. This may seem obvious, but clinicians may have an ambiguous attitude towards BCC and to a lesser extent SCC. On the one hand NMSC is considered a malignancy that requires an oncological approach (e.g.,

the cosmetic and functional outcome is inferior to complete removal) and on the other hand it may be trivialized because it is so common and its risk of metastasize is rare. This (subconscious) attitude may result in physicians saying phrases like 'it is skin cancer, but it is not a malignancy', 'BCC is the best of the bunch' or 'it is cancer, but it is not serious', which is confusing to patients. People with skin cancer should be informed about the necessity to treat NMSC adequately (including a long term follow up), the risk of metastases (exceptional for BCC and up to 10% for SCC)(13,14) ways to actively participate in the prevention of skin cancer (e.g., skin self examination, sun protection and chemoprevention) and reassured at the same time about the behavior of most NMSC.

Another predictor of skin related HRQOL after therapy was the general health of the treated individuals (measured by a comorbidity index and the SF-12). The importance of respondents` health status on patients HRQOL is recognized in the development of some HRQOL assessments (e.g., VAS for general health of the EuroQOL-5D). It is also confirmed by observations demonstrating that the presence of comorbidity affected the fear of developing a new BCC and is a significant predictor of HRQOL impairment in psoriasis patients.(2,15) Patients with a high comorbidity index score or those who indicated that health has a large impact on their QOL (i.e., low SF-12 scores) are less likely to notice an effect of skin cancer therapy than those who are in better health. Whether this is due to item bias (patients with the same level of skin related HRQOL impairment who are in poor health score several Skindex-16 items differently than those in better health), patients' inability to differentiate the impact of different diseases on their lives or a lack of the sensitivity of the measurement is not yet clear. But again, this population is in extra need of counselling prior to NMSC therapy to optimize its outcome.

The primary outcome of the study in this issue of the *Archives* is the mean Skindex-16 score of multiple assessments and each obtained Skindex-16 at time of assessment score is the mean of its three scales. This approach was chosen to minimize loss of data and based on the assumption that Skindex-16 scores did not change after 12 months, which is in contrast with other dermatological HRQOL studies that suggest that some concepts change in time as patients cope with and adjust to chronic conditions(2,15) Also, the proportion of patients who responded one, two or three times is unclear and a substantial loss of follow up may have affected the results of this study. Although it is stated throughout the result section that similar trends were detected for the three scales, the presentation of a total Skindex-16 score is

unfortunate because it does not allow to assess the effect of therapy on the emotion scale, which represents most NMSC induced variance of the Skindex-16 scores because it include two items that are highly relevant to skin cancer patients (i.e., how often patients have been bothered by `the persistence/reoccurrence` and `worry about spreading, getting worse, scarring and being unpredictable` about their skin condition). The later item is a composite question, which assesses multiple issues at once, providing important but non-specific information about the impact of NMSC. Moreover, from a theoretical perspective the use of a total score is inappropriate because it has no face validity, it was not formally tested and factor analysis have shown that the structure of the Skindex-16 is multidimensional (16). This implies that each dimension should be presented with separate scale score and that an overall score is suboptimal and should not be used as an interval measurement.

Although it is comprehensible from a methodological perspective that the authors only included new NMSC patients, the studyresults are not generalizable for all NMSC patients. A substantial proportion of patients with NMSC has a history of multiple (pre)malignant skin tumours and it would be interesting to investigate whether these patients experience NMSC differently than those with their first NMSC. Does a history of several NMSC enhance patients' anxiety of recurrence and subsequent cancers or are they more likely to psychologically adapt to living with NMSC than those with a first NMSC? Another interesting issue is the effect of patient education about NMSC on HRQOL and treatment satisfaction. It could be that patients appreciate more detailed information or that the proverb 'let sleeping dogs lie' applies and that it increases their worry and fear.

Interpretation of Comparisons

Statistical comparisons between HRQOL scores before and after an intervention should be interpreted with caution because a statistical significant difference does not always equal clinical significant difference. The requisites of comparing HRQOL scores are that nonparametric statistical techniques are used and that the measure is reliable (i.e., provide similar scores in patients with unchanged disease status at different time points), responsive to clinical change and that the meaning of obtained scores and differences are known. An aid in interpreting HRQOL scores is categorization of a continuous score in different levels of impairment. The authors

have created categories based on the tertile distribution of the mean scores, which is helpful in presenting data but is not a formal way of categorizing PRO' outcomes such as distribution based or banding techniques.(17) The minimal clinical important difference (MCID) informs clinicians that a difference in HRQOL scores observed after therapy is likely to have at least some clinical relevance to most patients. The authors assumed that the MCID of the total Skindex-16 score is 10 points because it was estimated to be about 10 points for each of the three scales. (8) Although with some limitations, the authors have presented the HRQOL data in such a way that statistical and clinical significance are likely to overlap.

Advantages of new Treatments

No statistical difference in HRQOL changes was detected between traditional excision and Mohs surgery of NMSC suggesting that patients primary concern is the excision of the malignancy. Although it is expected that a detailed examination of tumor margins in Mohs surgery would result in a more satisfied and reassured patient, which would be reflected in an improvement of HRQOL, the work by Chren et al suggests that patient do not appreciate it as much as expected. Also, undergoing a more complicated procedure such as Mohs surgery may affect patients' perception about NMSC differently than a traditional excision or ED&C. For now, the theoretical advantages of Mohs surgery compared to traditional excision such as a reduced recurrence rate, improved cosmetic outcome, detailed examination of tumor margins and cost effectiveness have not (yet) been confirmed from a physician, patient and regulatory perspective.(8,18,19) The advantage of Mohs surgery for NMSC concerning recurrence rate and HRQOL improvement may be more pronounced for high risk facial NMSC such as micronodular, morpheaform or recurrent BCC in the H-zone and after long term follow up (i.e., 5 years). This implies that there is no evidence to treat every (facial) BCC with Mohs surgery and that a strict patient selection is appropriate.

The recent trend in the care of NMSC is to use non or minimal invasive treatments such as photodynamic therapy (PDT) and imiquimod. The primary advantage of these options is to reduce the functional and cosmetic morbidity associated with the treatment of skin cancer. Interestingly, Chren and colleagues have demonstrated that ED&C, which can be considered a minimal invasive technique that may scar, did

not significantly improve HRQOL in NMSC patients. In part, this may be explained by the fact that patients' worry and fear do not substantially improve after therapies that do not 'physically' remove the skin cancer. Although the new minimal invasive techniques focus on the benefit of avoiding scars, patients' perceptions about the efficacy and safety of these therapies is not well documented and may not reassure patients as much as surgical excisions. Patients' beliefs about therapies warrant further investigation.

Sensible Resource Allocation

NMSC treatment is in the top five of most costly cancers and consumed about half a billion dollars annually in the US.(20) The costs of NMSC are likely to continue to grow due to an increasing NMSC incidence in the next decades.(21) In addition to clinical guidance, the effect of therapy on HRQOL can be used to justify the societal costs associated with NMSC therapy. Preference based HRQOL measures such as the EuroQOL-5D and the SF-6D are expressed in quality adjusted life years (QALY), which combines information about the level of HRQOL impairment and its duration (i.e., a QALY equals one year of full quality). Because of the relatively limited mortality of NMSC and HRQOL impact the costs per QALY for NMSC therapy may be high, especially for Mohs surgery. The high NMSC incidence warrants the development and use of cost-effective therapies to control the impact of NMSC on national health care budgets. To make an evidence-based decision in NMSC management well-performed HRQOL and pharmacoecomonic studies alongside randomized clinical trials are needed.(22)

Conclusion

Demonstrating HRQOL improvements after NMSC therapy and its predictors is important because it may affect treatment choice and outcome, identify patients in need for additional information, and allocation of health care budget. For now, the obvious advantages of Mohs surgery have not yet been confirmed from physicians', patients' and regulatory agencies' perspective and additional studies are warranted to study the effect of NMSC therapies.

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Chapter 10

General Discussion

General Discussion

The subject of this thesis is the investigation of the role of, and mechanism(s) underlying light fractionation in ALA-PDT for the treatment of non-melanoma skin cancer. In addition the role of evidence based medicine, guideline development and quality of life in clinical PDT are addressed.

Clinical Response to Light fractionated ALA-PDT

Since its introduction in the 1990's photodynamic therapy (PDT) using porphyrin pre-cursors is now widely accepted as an attractive treatment option for non melanoma skin cancer. It has also been applied to a variety of non-malignant skin diseases and is under investigation for the treatment of other malignant conditions in various organs. Initial high complete response rates (CR), well above 90%, for the treatment of superficial basal cell carcinoma (BCC) using ALA are reported. However long term clinical results have shown considerable variations, where CR has been found to be as low as 30%. This was a particular problem for nodular lesions. Clinically this led investigators to adopt approaches to enhance clinical response. The minimal side effects of PDT using topical ALA or its derivatives mean that repeat treatments were a simple option. Indeed the recommended regime for the PDT using methyl-5-aminolevulinate (MAL) is two treatments one week apart. Further subsequent treatment sessions are often performed. Our approach however has been to first optimize PDT after a single application of porphyrin pre-cursor.

The results presented in **chapters 2 and 3** clearly show that light fractionated ALA-PDT can significantly improve the response rates in the treatment of sBCC and Bowens disease. While follow up remains short (12 months) we believe that all dermatologists, both within and outside academic hospitals should consider to use this type of light fractionated regime for patients with superficial BCC and Bowens disease. The rising incidence of non melanoma skin cancer illustrates that optimised treatment protocols are relevant not only for dermatologist but for wider health policymakers.

Our sBCC study included clinically diagnosed secondary BCC, just as occurs in routine clinical practice. Clinical diagnostic accuracy in a dermatology university faculty has been shown to be approximately 70% for BCC (Presser and Taylor,

1987). Therefore a small number of nodular BCC's were included due to these treated lesions. Analysing our results, and assuming a similar accuracy as Presser and Taylor predicts the three recurrent or non-responding nodular BCC's in the light fractionated illumination arm, represents a significantly lower number than expected (P = 0.0003 Fisher's exact test). This is an indication that clinically light fractionated ALA-PDT may lead to deeper tissue damage. We have previously shown that the two-fold illumination leads to deeper histological damage in pre-clinical models (De Bruijn *et al*, 1999; Robinson *et al*, 2003). Clinically, in sBCC and Bowens disease a more severe acute response is observed following light fractionated PDT, compared to a single illumination. Importantly however healing times remain relatively short and the cosmetic outcome, in almost all cases, is good or excellent. The longer overall treatment time and the increase in acute response may have lead to a more painful therapy. However we did not find a significant increase in the need for pain relief in our clinical studies and most patients tolerated the treatment without any form of pain medication. For Bowens disease an extra argument in favour for PDT can be found in the fact that these lesions often occur on so called 'difficult to treat' areas as lower legs and face .For patches in these areas, and in particular for the larger ones, there is a need for an alternative to surgery.

The Mechanism behind the Increase in Response

One aim of this thesis was to investigate the mechanism underlying the increase in response of tissues to light fractionation and to confirm that our pre-clinical mechanistic studies were representative of aspects of clinical PDT of non-melanoma skin cancer. We investigated the process of PpIX photobleaching, in which the photosensitiser is progressively destroyed during clinical ALA-PDT. In earlier work photobleaching has been proven to limit the PDT dose which can be delivered in a single illumination at fixed fluence rate and this was our original rationale for investigating the use of extended dark intervals between light fractions. As described below we have found that the overall amount of PpIX utilised seems not to be directly related to the increase in response that we observe. Clearly the mechanism behind the increased response to fractionated ALA-PDT is complex and the data presented here are part of an ongoing investigation. We have however confirmed that the kinetics of PpIX fluorescence photobleaching and re-synthesis

are very similar in animal models and in the clinic.

In **chapter 4** we investigated the distribution of PpIX fluorescence in sBCC using a surface measurement and microscopic PpIX fluorescence. We found wide variations in the average intensity of PpIX fluorescence in biopsies of control lesions and in biopsies of lesions 2 hours after illumination with 20 J cm^{-2}. We also found wide variations in fluorescence-intensity within individual sections of biopsies. Given these large standard deviations on average fluorescence-intensities we found a reasonable correlation between the two different methods of performing fluorescence microscopy. In the described study we have shown for superficial BCC, where ALA penetration and transport are unlikely to be limiting factors, there remain regions of tumour that accumulate and re-synthesize relatively small amounts of PpIX. It is also clear that regions of low PpIX fluorescence accumulation are not located at the base of superficial tumours and high PpIX fluorescence is often observed at the periphery of tumour islands at the base of the tumour. Availability of ALA therefore is unlikely to induce these differences. We have shown that highly proliferating normal and tumour cells synthesize relatively more PpIX and that this corresponds with the nuclear stain Ki-67 a marker for cellular proliferation. The results of this study also have illustrated the complexity of using surface fluorescence detection as a diagnostic procedure. Our results suggest a limited value in the discriminating capacity by using PpIX fluorescence and this is certainly an area of interest in the future.

Our mechanistic investigations also included some apoptotic markers. The role of the Bcl-2 protein family in PDT is not fully determined but its seems to play an important role in the response of ALA-PDT in general. In BCC the presence of Bcl-2 and Bax has been described in literature. We were able to confirm this presence in our study (**chapter 5**). In literature expression of apoptosis markers is predominantly not investigated in vivo and not in BCC. Therefore it may be not applicable to sBCC after ALA-PDT but the results support strongly mitochondrial apoptosis is involved in PDT mediated cell death. Our findings, an increased expression of Bax after a small dose ALA-PDT suggest a relatively large cell population Bax- positive cells in BCC is prone to apoptosis. After a single full dose still Bax expression is found. Due to small number investigated we modestly conclude Bax plays a role. Caspase 3 , the executor caspase has been described early after PDT. A rapidly increasing expression of caspase 3 is seen in almost all specimens in our data 2 hours after a single small dose ALA-PDT. This suggests early apoptosis is induced by ALA-PDT

and confirms the first fraction's importance for the ultimate complete response of the BCC to ALA-PDT. In future, serial observations in time and quantitative analysis may contribute to a better understanding of the apoptosis induced by ALA-PDT in human BCC.

In **chapter 6** we investigated the role of light fractionation using methyl aminolevulinate (MAL) in normal mouse skin. This is particularly interesting since it is MAL that is the registered porphyrin pre-cursor for the treatment of AK, superficial BCC and Bowens disease. Contrary to our expectations, we have found significant differences for MAL and ALA. Light fractionation proved not to be effective using MAL. This is despite the fact that pharmacokinetics of PpIX fluorescence in normal mouse skin during the 4-h application period are identical for both ALA and MAL and the rate and extent of PpIX photobleaching after each light fraction for ALA or MAL are almost identical. One critical difference between ALA and MAL, and other ester derivatives of ALA, is their tissue distribution due to their different biophysical and biochemical characteristics. After topical application of MAL, PpIX fluorescence is retained at the application site whereas after ALA application PpIX fluorescence is also measured at distant sites in the skin. ALA is distributed more systemically after topical application whereas MAL is not. This seems to be a clear indication that the tissue distribution of PpIX (mediated by the different prophyrin-precursor) plays a significant role in mechanism behind the response of tissue to light fractionated ALA-PDT. This is clearly an area for detailed future investigations.

Evidence based Medicine, Guidelines and QOL

We strongly support the idea of evidence based medicine and this was the basis for the reaming chapters of the thesis. We appraised the existing clinical guidelines in dermato-oncological care using the European standard 'the AGREE instrument'.(**chapter 7**) Dermatological clinical guidelines have good quality but – as always- improvements can be made. Clear formulation and participation of all involved users can be particularly improved. An extra argument in favour of the AGREE instrument is, in our opinion, its recipe function in the development phase of a guideline. In respect to allocation of sources collaboration and consensus lead to more uniform clinical care, which is desirable for standard care. We like to note every patient is an individual and needs personal attention of his/her dermatologist.

The rapidly rising incidence of non melanoma skin cancer and its burden on society attributes to the need of dermatological evidence based care.

An aspect of social impact is expressed by patient reported outcomes. The last chapter (**chapter 9**) is an editorial in answer to a study investigating improvement of health related quality of life (HRQOL) after–treatment of non melanoma skin cancer. Surgery, Mohs' micrographic surgery and electro-dissection & coagulation were the involved treatments. The most important factor was the HRQOL before treatment in predicting the HRQOL after treatment. HRQOL is a fairly unexplored field in skincancer treated patients. Demonstrating HRQOL improvements after NMSC therapy and its predictors is important because it may affect treatment choice and outcome, identify patients in need for additional information, and allocation of healthcare budget. This is a field to be explored in future. Patients expectations and experiences should be two of the leading themes and should be accommodated in the choices associated in dermato-oncology.

Light fractionated ALA-PDT in routine Clinical Practice

Light fractionated ALA-PDT is now used in daily practice in Erasmus MC in Rotterdam for AK, sBCC, 'superficial growing" nBCC and for Bowens disease (**chapter 8**). The complete response-rates after a follow up of two years are concordant with our published clinical studies in which a follow up period of 12 months was described. For AK a complete response-rate of 98% was achieved. In all treated sBCC's 97% responded. For Bowens disease a complete response-rate of 84% was achieved, a result comparable to results in literature. And for nBCC a complete response-rate of 80% was found. The Bowens disease lesions which did not respond completely at first treatment session were retreated and showed a complete response after the second treatment, that is sustained for at least 12 months.

Future Perspectives

The follow up in our clinical studies is limited to 24 months. In oncology 5 years follow up rates are considered to be the gold standard. While we obviously

intend to follow these patients for 5 years we expect no great reduction in response-rate of our fractionated illumination scheme. Basset-Sequin et al have shown that for MAL-PDT response rates have been shown to be more or less equal after 48 and 60 months follow up. Our results in nodular BCC raise a range of questions and give weight to the need of optimization of PDT for these lesions or to more accurately define which lesions are suitable for treatment with PDT. Nodular BCC does respond, but in all studies using ALA and MAL this seems to be limited. More research is needed to define criteria according to which lesions are suitable for ALA-PDT using light fractionation. Prior curettage and repeated optimized light fractionated ALA-PDT is a clear treatment option.

Chapter 11

Summary

(English and Dutch)

Summary

Chapter 1 is an introduction to this thesis "ALA-PDT; the treatment of non melanoma skin cancer re-illuminated" . In the first part the reason for concerns about non melanoma skin cancer and its treatment is described. The incidence of BCC is growing rapidly and this is leading to an important problem in healthcare. The etiology of non melanoma skin cancer is primarily behavioral and related especially to exposure to sunlight. Current treatment modalities are surgery, topical chemotherapy, cryotherapy, immuno-modulating therapy, curettage & electrodessication, radiotherapy and photodynamic therapy. Phototherapy in dermatology is an ancient modality. This in also illustrated in a historical overview. Furthermore the principles of PDT are described in the introduction. In ALA-PDT the prodrug ALA is applied and leads to the synthesis of the true photosensitiser PpIX which leads to production of reactive oxygen species such as singlet oxygen by absorption of light. Singlet oxygen is responsible for the destruction of (tumor-)cells. The important factors in ALA-PDT, porphyrin synthesis, PpIX distribution, light and photophysics, photochemistry, photobleaching, photobiology are briefly explained.

ALA (and MAL) PDT have their limitations and therefore a need for optimization is recognized. Optimizing ALA-PDT by light fractionation in animal studies and a first pilot study are the base for the clinical study described in **chapter 2**. Here a comparative study of 505 superficial BCC's treated with ALA-PDT either single illumination (75 Jcm^{-2}) or two-fold illumination (20+80 Jcm^{-2} separated by a two hours dark interval) is descibed. The complete response to a two fold illumination is statistically significant higher than to a single illumination (CR 97% vs 89%, $p<0.002$) at a minimum follow up of 12 months. Most patients did not require any form of pain relief. In almost all cases the cosmetic outcome was regarded good to excellent by patients and physicians. In **chapter 3** a pilot study in 50 patches of Bowens disease is described. The same treatment parameters as in the sBCC-study were used. Also in Bowens disease the response to a two-fold illumination is higher than to a single illumination, 88% vs 80% CR at a minimum follow up of 12 months. An argument in favor for PDT in the treatment of Bowens disease is the relatively short healing time and excellent functional and cosmetic outcome. This is especially an advantage on so called difficult to treat areas such as lower leg and face.

Besides clinical studies we studied the PpIX kinetics in sBCC in relation to our earlier work. In **chapter 4** the PpIX fluorescence measured at surface and microscopically is described. We found a wide variation between biopsies as well as within biopsies. A relatively good correlation was found between the two techniques we used. Both control lesions and treated lesions were included. Biopsy was taken at 6 hours after ALA application or at 2 hours after 20 Jcm^{-2} ,i.e. 6 hours after ALA application. The specimens were immunohistochemically stained for the proliferation marker Ki-67. PpIX fluorescence pattern follows the Ki-67 positive stained cells. PpIX fluorescence confirmed relative re-synthesis in the dark interval in a two-fold illumination scheme consistent with our earlier work. In our search to enlighten the mechanism of ALA-PDT and especially the increased response to fractionated ALA-PDT we also investigated cell death. Cell death in (ALA)-PDT is due to necrosis, apoptosis and an immunological response. We performed immunohistochemical stainings for the Bcl-2, Bax, Caspase 3 in single illuminated and two fold illuminated sBCC's which is described in **chapter 5**. Biopsies were taken after a full single dose (n=10), just before the second illumination in the twofold scheme(n=25) and after ALA application only (control, n=7)). One sBCC was serial biopted in time. (3 biospies). The Bcl-2 protein family is recognized to play a role in the apoptotic process induced by PDT. Caspase 3 can be regarded as the executor caspase in apoptosis. Early after a full dose ALA-PDT as well as after only a small dose, apoptotic markers show altered expression indicating apoptosis is occurring. We confirm the idea apoptosis induced by the mitochondrial pathway plays a role in cell death in ALA-PDT in sBCC.

Because of the increased use of MAL in dermatological practice we investigated the response of fractionated MAL-PDT in normal mouse skin. These results are described in **chapter 6**. We expected to gain efficacy by fractionating the illumination in correlation to the increase response we gained by fractionated ALA-PDT. Our results, however, show that fractionated illumination does not enhance the efficacy of PDT using MAL as it does using ALA despite the comparable fluorescence intensities at the end of the first light fraction and at the start of the second light fraction. Only the initial rate of photobleaching was slightly greater during ALA-PDT although the difference was small. Our data suggest that the distribution of MAL and ALA in tissues, and therefore the site of PDT induced damage, is an important parameter in the mechanism underlying the two-fold illumination scheme.

In **chapter 7** we are back to clinical dermatology. The quality of guidelines in dermato-oncology are described. We appraised the available (till 2006) clinical

guidelines (n=20) according to the AGREE instrument. The AGREE instrument is the result of an European collaboration. The domains in the AGREE instrument concern especially the methods of development of guidelines. The score according to AGREE can be improved by explicitly mentioning the different items. Guidelines in dermato-oncology appeared to be of reasonable quality. In a comparison of guidelines before and after publication of the AGREE instrument improvements are seen concerning the clarity. As clinical guidelines are regarded to be an important instrument to improve quality of care, improvements are needed.

The results of fractionated ALA-PDT in Erasmus MC in Rotterdam for AK, sBCC, nBCC and Bowens disease (in a total of 552 lesions) are described in **chapter 8**. After a follow up of 2 years high complete response-rates are achieved using a fractionated regime of 20 + 80 J cm^{-2} separated by a 2 hours dark interval. For sBCC the CR at two years was 97%, for AK 98%, for Bowens disease 84 % and for nBCC 80%. The results of Bowens disease is comparable to other results in literature as to the result of surgery suggesting ALA-PDT is a good treatment option but improvements are desirable. Also in nodular BCC the CR rates are subject to optimization. A sub-analysis of the results of all lesions larger than 2 cm showed CR at two years of 89% for all lesions (n=57). Cosmetic outcome was good to excellent in almost all cases. The obtained results should be included in future guidelines.

Chapter (**9**) is an editorial about health related quality of life. In addition to CR rates, disease free interval and other physician based parameters patient reported outcomes are important. Skin cancer specific HRQOL questionnaires such as the Skin Cancer Index have been introduced recently. A better understanding and documentation of patients` views is helpful in improving patient-physician interaction, skin cancer care, treatment outcome and the selection of patients in need of additional pre-treatment counselling. Demonstrating HRQOL improvements after NMSC therapy and its predictors is important because it may affect treatment choice and outcome, identify patients in need for additional information, and allocation of health care budget. For now, the obvious advantages of Mohs surgery have not yet been confirmed from physicians', patients' and regulatory agencies' perspective and additional studies are warranted to study the effect of NMSC therapies.

SAMENVATTING

Hoofdstuk 1 van dit proefschrift ' ALA-PDT de behandeling van (niet-melanoom) huidkanker opnieuw belicht' is een inleiding. Hierin wordt beschreven waarom het nodig en noodzakelijk is aandacht te besteden aan huidkanker en de behandeling hiervan. De incidentie (het voorkomen) van het basaalcelcarcinoom stijgt snel en zal in de nabije toekomst leiden tot een probleem in de gezondheidszorg. Huidkanker wordt voornamelijk veroorzaakt door zonlicht blootstelling. De huidige behandelmogelijkheden zijn chirurgie, lokale chemotherapie, cryotherapy (bevriezen), immunomodulerende (weerstand-beinvloedende) therapie, curettage en electrodessicatie (weghalen met een scherpe lepel en nabranden), radiotherapie en fotodynamische therapie (PDT).

Behandeling met licht bestaat al heel lang in de dermatologie. Dit wordt geïllustreerd in het historisch overzicht (en tabel). De principes van fotodynamische therapie worden in de inleiding beschreven. Bij gebruik van ALA-PDT wordt een voorloper van protoporphyrine IX (PpIX) aangebracht.PpIX is een lichtgevoelige stof die bij belichting zorgt voor de productie van reactieve zuurstofmoleculen. Deze reactieve zuurstofmoleculen zorgen voor schade en vernietiging van de (kanker-)cellen. Belangrijke factoren die een rol spelen bij ALA-PDT zoals de aanmaak van PpIX, de verdeling van PpIX , de eigenschappen van licht, her-verbruik van PpIX, de natuurkundige, biologische en chemische processen van PDT worden kort uitgelegd. Er zijn beperkingen van ALA-PDT en MAL-PDT zodat verbetering, optimalisering gewenst is. In dierstudies en in een eerste beperkte klinische studie is aangetoond dat fractionering van de belichting kan leiden tot een beter resultaat. Dit was de basis voor de studie die is beschreven in **hoofdstuk 2** van dit proefschrift. Een vergelijkende studie naar het resultaat van behandeling van oppervlakkig groeiende basaalcelcarcinomen (sBCC) tussen een enkelvoudig (75 Jcm^{-2}) en een tweevoudig belichtingsschema (20+80 Jcm^{-2} .met een twee uur durende donkere pauze tussen de belichtingen). Het resultaat na minimaal 12 maanden van de tweevoudige behandeling is statistisch significant beter dan van de enkelvoudige belichting.(97% genezen in vergelijk met 89% genezen. $p<0.002$). De meeste patiënten konden de behandeling goed verdragen, zij hadden geen pijnstilling nodig. Het cosmetisch resultaat werd goed tot excellent beoordeeld door de patiënten en behandelaars.

In **hoofdstuk 3** wordt een kleine studie beschreven naar de behandeling van M Bowen. Dezelfde behandelschema's als in de sBCC studie werden gebruikt. Ook bij M Bowen heeft een tweevoudige belichting een beter resultaat. Na 12 maanden was in de enkelvoudige groep 80% genezen tegenover 88% in de tweevoudig belichte groep. Een extra reden om M Bowen te behandelen met ALA-PDT is de korte genezingstijd na de behandeling. Dit is met name een voordeel voor die afwijkingen die voorkomen op lastig te behandelen plaatsen zoals het gelaat of het onderbeen.

Naast klinische studies hebben wij ook onderzoek gedaan naar de PpIX kinetiek in relatie tot eerder dierexperimenteel onderzoek. In **hoofdstuk 4** worden de resultaten beschreven van fluorescentie onderzoek aan het oppervlak gemeten en microscopisch gemeten. Een goede correlatie werd gevonden tussen deze twee methoden om PpIX fluorescentie te meten. Zowel behandelde sBCC's als controle biopten werden onderzocht. Zes uur na het aanbrengen van ALA werd een biopsie gedaan of 2 uur na een eerste belichting (20 Jcm^{-2}),dat is ook 6 uur na het aanbrengen van ALA. De biopten werden immunohistochemisch gekleurd voor Ki-67 een marker voor proliferatie. Het fluorescentie patroon komt goed overeen met de positief aankleurende cellen voor Ki-67. De PpIX fluorescentie bevindingen bevestigen het eerder gevonden feit dat er relatief nieuw PpIX aangemaakt wordt in het donkere interval tussen de belichtingen in een tweevoudig behandel schema. In onze zoektocht naar een verklaring van het werkingsmechanisme van ALA- PDT en met name van een gefractioneerde behandeling hebben we ook onderzoek gedaan naar het patroon van celdood. Bij ALA-PDT gaan de cellen dood door necrose (versterf) en/of door apoptose (geprogrammeerde celdood zonder resten) en door een immunologische respons.Immunohistochemisch kleuringen werden verricht naar Bcl-2, Bax en caspase 3 in enkelvoudig belichte sBCC's, in tweevoudig belichte sBCC's en in controle sBCC's. Deze resultaten worden beschreven in **hoofdstuk 5**. Biopten werden genomen 6 uur na het aanbrengen van de ALA (controle, 7 biopten), 2 uur na een enkelvoudige belichting (10 biopten) of net voor de tweede belichting in het tweevoudig belichtingsschema (25 biopten). Bij één patient werd een sBCC driemaal gebiopteerd. Eiwitten van de Bcl-2 familie worden geacht een rol te spelen bij de celdood door apoptose bij ALA-PDT. Caspase 3 kan worden beschouwd als een uitvoerder in apoptotische celdood. Snel na een enkelvoudige behandeling worden kenmerken gevonden van apoptose, zoals een veranderde uitdrukking van Bcl-2 en Bax eiwitten. Onze resultaten bevestigen dat apoptose.

aangezet via het mitochondrium (energiefabriek van de cel) in de cel een rol speelt bij de celdood door apoptose bij ALA-PDT.

Omdat in Nederland MAL in toenemende mate wordt gebruikt bij PDT hebben we ook onderzoek gedaan naar het effect van een gefractioneerde belichting bij MAL-PDT. In **hoofdstuk 6** zijn de resultaten van dit dierexperimenteel onderzoek bij muizen beschreven.Wij hadden verwacht een groter effect te bereiken door fractionering van de belichting zoals wij een groter effect verkregen bij gefractioneerd ALA-PDT. Echter de effectiviteit van MAL-PDT neemt niet toe zoals bij ALA-PDT hoewel de fluorescentie intensiteit bij beide sensitisers (lichtgevoelige stoffen of hun voorloper) na de eerste belichting en juist voor de tweede belichting vergelijkbaar zijn. De mate waarin PpIX verbruikt wordt bij ALA-PDT is in eerste instantie iets sneller dan bij MAL-PDT. Dit is echter een te klein verschil om het grote verschil in PDT schade / resultaat te verklaren. De verkregen gegevens suggereren dat de verdeling van ALA en MAL in het weefsel en daarmee de plaats van de schade door PDT een belangrijke rol speelt in de toegenomen schade bij gefractioneerde ALA-PDT.

In **hoofdstuk 7** wordt onderzoek in de klinische dermatologie beschreven. De kwaliteit van richtlijnen voor de behandeling van huidkanker wordt beschreven. De (20) richtlijnen die beschikbaar waren tot 2006 werden beoordeeld met het AGREE instrument. Dit is tot stand gekomen in samenwerking met een aantal Europese landen en betreft met name de kenmerken en methodes van totstandkomen van een richtlijn. De score die verkregen wordt met het AGREE instrument kunnen worden verbeterd door het expliciet noemen van de verschillende onderdelen van een richtlijn. De kwaliteit van dermatologische richtlijnen voor de behandeling van huidkanker is redelijk gebleken. Richtlijnen gepubliceerd na de publicatie van het AGREE instrument zijn helderder geformuleerd dan die tevoren zijn gepubliceerd. Omdat richtlijnen belangrijk zijn voor een goede kwaliteit van zorg blijven verbeteringen wenselijk.

In **hoofdstuk 8** worden de resultaten van de gefractioneerde ALA-PDT voor actinische keratose, sBCC, nodulair BCC en M Bowen beschreven. Na twee jaar zijn goede genezingspercentages verkregen na behandeling met het tweevoudig belichtingsschema van 20 + 80 J cm^{-2} met een twee uur durend donker interval van totaal 552 afwijkingen. sBCC reageerde in 97% van de gevallen, AK in 98%, nBCC in 80% en M Bowen in 84% met een genezing. De resultaten bij M Bowen zijn vergelijkbaar met de in de literatuur beschreven resultaten van PDT en chirurgie, maar maken duidelijk dat verbetering gewenst is. Ook een verbetering in de genezing van

nBCC is gewenst. Een analyse van de 57 afwijkingen groter dan 2 cm voor BCC's, AK en M Bowens gaf in 89% een genezing na 2 jaar. Het cosmetisch resultaat was in nagenoeg alle gevallen excellent.Onze resultaten zouden moeten worden opgenomen in toekomstige richtlijnen voor de behandeling van niet-melanoom huidkanker.

Hoofdstuk (9) bestaat uit een editorial over kwaliteit van leven die gerelateerd is aan gezondheid. Naast genezingpercentages, ziektevrije overlevingstijd en andere doktergerelateerde parameters zijn de door de patiënt gerapporteerde uitkomsten belangrijk. Vragenlijsten over kwaliteit van leven die specifiek huidkanker gerelateerd zijn, zijn recent ontwikkeld. Een voorbeeld is de Skin cancer index (huidkankerindex). Beter begrip en vastlegging van de mening van patiënten kan behulpzaam zijn in de arts-patiënt relatie, de huidkanker behandeling en de selectie van patiënten die aanvullende begeleiding of informatie nodig hebben. Het aantonen van effecten op de kwaliteit van leven voor en na een behandeling van huidkanker is belangrijk. Deze effecten kunnen belangrijk zijn voor patiëntselectie, informatiebehoefte van patiënten, therapiekeuze en resultaat evenals voor de verdeling van budget en mogelijkheden in de gezondheidszorg. Tot heden zijn de voordelen van Mohs' micrografische chirurgie, een speciale operatietechniek voor de behandeling van huidkanker niet bewezen vanuit patiënt perspectief. Aanvullende onderzoeken hiernaar zijn nodig.

Curriculum vitae

Ellen Regina Margaretha de Haas werd in 1964 op 4 april - de verjaardag van haar moeder - geboren in Amersfoort. Haar kinderjaren werden in Soesterberg en Schöppingen doorgebracht met veel buiten spelen en veel plezier. In 1974 verhuizen de Hazen naar Utrecht waar zij naar het Christelijk Gymnasium gaat en daarna geneeskunde studeert.In januari 1990 begint zij haar medische loopbaan als arts-assistent in een huisartsen praktijk. Tijdens deze periode maakt zij – als getuige – kennis met het recht.

Na een periode van algemene ervaring als wissel- arts assistent interne, gynaecologie en chirurgie in de Gelderse vallei in Wageningen en een arts assistentschap radiotherapie in de Daniel den Hoed Kliniek in Rotterdam begint zij in 1992 aan de huisarts opleiding in Utrecht. Hierbij werd een deel gewerkt in Olst en in Nieuwegein. In deze tijd komt de vraag naar kennis van het recht weer boven en besluit zij, in haar vrije tijd, rechten te gaan studeren. Tijdens het werk als huisarts in opleiding blijkt zij te weinig te weten van de huid, dit gebrek wordt verholpen door een extra stage dermatologie in het Academisch Ziekenhuis in Utrecht bij prof. Willem van Vloten. Daar maakt zij kennis met het prachtige vak dermatologie en één zeer aardige dermatoloog. Na het beëindigen van de huisartsopleiding - waar je aan begint maak je af - werkt zij enige tijd met veel plezier als waarnemend huisarts in verschillende praktijken. Langdurig in Bruinisse en Huizen. De dermatologie en de dermatoloog hebben haar hart gestolen.Op 1 april 1997 kan worden gestart met de specialisatie tot dermatoloog bij prof. Willem van Vloten en prof. Carla Bruijnzeel. In september 1997 trouwt zij met Frederik de Wit. In 1998 leidt haar hobby recht tot het behalen van de meester titel. (Nederlands recht in het bijzonder privaat recht, gezondheidsrecht). Een uitvloeisel van deze hobby is haar voorzitterschap van de taakgroep dossier en bewaartermijn in het wets- evaluatie project van de WGBO (KNMG ism WVS). Sedert januari 2002 versterkt zij de equipe van prof. Martino Neumann in het Erasmus MC. Dermato-oncologie wordt haar speerpunt, het onderzoek naar optimalisering van ALA-PDT krijgt haar aandacht. Tijdelijk (2005) verhuist zij 2 dagen per week naar Eindhoven om opgeleid te worden in de Mohs chirurgie, met enthousiasme, door Dr Judith Ostertag en Dr Gertruud Krekels. Opgedane kennis wordt in Rotterdam in praktijk gebracht.

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